

A Laboratory Guide to the Mycoplasmas of Human Origin

York E. Crawford



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**NAVAL MEDICAL RESEARCH UNIT No. 4
GREAT LAKES, ILLINOIS**

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Naval Medical Research Unit No. 4
Great Lakes, Illinois (60088)

A LABORATORY GUIDE TO THE MYCOPLASMAS OF HUMAN ORIGIN
(Second Edition)

By

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* Deceased



Dedicated to the Memory of

YORK E. CRAWFORD

1918—1971

Mr. York E. Crawford may be called one of the pioneers in the relatively new science of Mycoplasma, and was a widely recognized authority in his field. When the Mycoplasma Division was established in 1961, he was appointed Chief of that division, and continued in that capacity until his untimely death in March, 1971.

Although the mycoplasmas were well established in scientific literature by this time, there was no single source of laboratory information. Mr. Crawford soon saw the need for a comprehensive laboratory manual describing in detail the techniques used to isolate and identify these elusive organisms, and began to assemble and write such a manual, which was published in 1966. The immediate and widespread interest it created was greater than had been anticipated, and it was reprinted to meet the demand. By 1969, Mr. Crawford had begun the painstaking task of updating and revising the manual. At the time of his death, he had accumulated a wealth of new material and had completed the first draft of this, the Second Edition. His co-workers completed the organization and editing of his draft. This edition, insofar as possible, appears exactly as York had intended it, including his own dedication to his friend and mentor, Dr. John J. Robinson.



Dedicated to
JOHN J. ROBINSON, B.A., M.S., M.D.
who fostered the author's interest in the
mycoplasma and L-Phase of bacteria

PREFACE

This laboratory handbook, "A Laboratory Guide to the Mycoplasmas of Human Origin" is an updated version of the earlier printing of 1966. The progress of recent years, such as improved media, new serological techniques, advances with T-strains, newly identified species, and easier methods of cultivation have prompted this revision. Much of the information compiled in this booklet emerged from the "Second Conference on the Biology of the Mycoplasmas" held in New York City in 1966, where the need for standard procedures was clearly expressed. Since this conference a steady stream of research papers has provided a rich source of subject material on methods for the isolation and study of mycoplasmas originating from man.

In the contents which follow, methods for serological testing have been included. In outlining these step-by-step procedures it is hoped that the reader will not hesitate to consult original references and texts to aid his comprehension of the principles involved. In keeping with laboratory guides of this type, the theoretical and clinical aspects of the organisms under discussion are briefly introduced but intentionally held to a minimum. Sources of supplies and equipment for mycoplasma work will be found at the end of the booklet. The references are placed at the end of each section for the convenience of the reader. It is hoped that the methods outlined herein will be of assistance to the technician, microbiologist, student, instructor, or research worker who finds himself confronted with the specialized problems of this field. The clinical laboratory, charged with diagnostic services, should especially benefit from the material which follows.

The author is indebted to Captain Robert O. Peckinpaugh (MC) USN and Commander Wayne E. Frazier (MC) USN for encouraging and supporting the compiling, writing, and printing of this manual. The further encouragement of Dr. Harry S. Morton is deeply appreciated. A number of persons on the staff, and members of the Mycoplasma Research Division of the Naval Medical Research Unit No. 4 have made significant contributions to the contents of this booklet. William H. Kraybill and George M. Allen have been indispensable in their evaluation of many of the techniques outlined herein. The hours of typing required to prepare this guide, and much of the editorial work is due to the skills and experience of Mrs. Lula Duggan, Mrs. Patricia Warren and Mrs. Marilyn Tervola. The true contributors are those workers whose names may be found in the cited literature.

The revision of this manual was interrupted by the untimely death of the author on 12 March, 1971. It was completed by William H. Kraybill with editorial assistance by David W. Beno and Robert P. Nalewaik.

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HISTORICAL SKETCH

The smallest living microorganisms that grow on artificial media measure from 125 to 250 m μ and belong to the family Mycoplasmataceae. For many years the members of this group were called "pleuropneumonia-like organisms," and were abbreviated "PPLO." These terms arose from the fact that the prototype species, and earliest isolate, was recovered from cattle suffering from a condition known as contagious bovine pleuropneumonia (1). The nomenclature became confused when other names were proposed by various workers who encountered organisms with similar characteristics. The disorder in nomenclature was resolved by placing all of them in a single genus, naming it *Mycoplasma* (fungus form) and listing the organisms in Bergey's manual (2). In 1971, however, the Subcommittee on the Taxonomy of Mycoplasmatales approved a proposal by Edwards and Freundt to establish a new genus, *Acholeplasma*, and a new family, *Acholeplasmataceae*, for strains like *Mycoplasma laidlawii* which do not require sterol for growth (3, 4).

The first isolate to be obtained from a human subject was reported in 1937 by Dienes and Edsall who recovered a typical strain from an infected Bartholin's gland (5). That the genital tract serves as a habitat was verified over the next 30 years by reports from numerous authors. The presence of mycoplasmas in another site of the body was reported in 1951 by Morton, Smith, Williams and Eickenberg (6) who were first to demonstrate that human saliva often contained mycoplasmas. Smith and Morton then demonstrated that typical strains were also obtainable from the human oropharynx by throat swab technics (7). In 1953, Dienes and Madoff recovered mycoplasmas from tooth scrapings and from tonsillar exudates (8). Further stimulation for study was provided by Card, who demonstrated by serological methods that mycoplasma originating from the buccal cavity differed from the genitourinary strains (9). The atypical "T-strains," first recognized by Shepard, have rekindled interest in the role of mycoplasmas in nongonococcal urethritis (10). Reports of a "T-strain" role in spontaneous human abortions which further expands the scope of their possible significance, should stimulate investigations of other genitourinary conditions of unknown etiology (11).

A filterable agent, isolated from a patient with primary atypical pneumonia by Eaton in 1944, was shown to be a mycoplasma 18 years later (12, 13). This work was of monumental importance to the

field because of the later demonstration that the agent of Eaton was a member of this genus, and a pathogen. Eaton observed that his filterable agent multiplied in eggs, produced pneumonic lesions in cotton rats, and was sensitive to tetracycline compounds. Liu, utilizing immunofluorescence, devised a serological test for the quantitative measurement of antibodies thus making serological testing a feasible procedure for the study of Eaton Agent pneumonia (14). Clyde observed Eaton Agent particles in tissue cultures and suggested its relationship with the genus *Mycoplasma* (15). Two investigators working in England, Marmion and Goodburn, visualized very minute coccid forms in the bronchial mucosal area of hamsters infected with the Eaton Agent (16). Coupled with other observations, notably sensitivity of the agent for gold salts, they suggested further that the Eaton Agent was a mycoplasma. At this stage of knowledge, Chanock, Hayflick and Barile were able to grow Eaton Agent into colonies of mycoplasma on solid, artificial medium (13). Now classified as *Mycoplasma pneumoniae*, and well known for its association with cold agglutinins-reactive pneumonia, this microbe has received the attention of many workers throughout the world. At Great Lakes, Illinois, work in this direction was intensified by the Naval Medical Research Unit No. 4 when admissions for pneumonia at the U.S. Naval Hospital reached 2,500 cases from June, 1960 to May of 1961. The experiences gained with various technical procedures during ten years of work was thought worthy of documentation and provides the foundation for the sections which follow.

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HISTORICAL SKETCH

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MYCOPLASMAS ISOLATED FROM MAN

Fifteen species of mycoplasmas isolated from various sources are placed within the genus *Mycoplasma* in the seventh edition (1957) of Bergey's Manual (1). Only 3 of these are of human origin. *Mycoplasma hominis* is presented as a single species with subtypes 1 and 2; however, in 1965 *M. hominis* type 2 was withdrawn from this position because carefully performed serological analyses showed it was actually *Mycoplasma arthritidis*, a species isolated from rats (2). Since the printing of Bergey's 7th edition a total of 35 named species are now listed, and 5 of these are new and not listed in Bergey's Manual as being of human origin (3). The first, *Mycoplasma pneumoniae*, already has been mentioned. In 1963 Herderschee, Ruys, and Van Rhijn, described but did not name a second species (4). The same new species was described by Clyde, and named *Mycoplasma pharyngis* in April of 1964 (5). The "pharyngis" designation seemed appropriate because of a predilection for the pharyngeal region. In July 1964, Taylor-Robinson and associates, reporting on the same organism, suggested that it be called *Mycoplasma orale* (6). An attempt to resolve the dilemma was made by the Subcommittee on the Taxonomy of Mycoplasmatales which convened in Mexico City in August 1970. They submitted a request for an opinion to the Judicial Commission that the name *Mycoplasma pharyngis* be rejected and that the name *Mycoplasma orale* type 1 be retained. The Judicial Commission decided not to act on the case since the name "pharyngis" was not validly published, because the publication, presented in abstract form, was not accompanied by a description (7). The third newer species, also recovered from the oropharynx, was next described by Taylor-Robinson, Fox, and Chanock (8). The name suggested for this organism was *Mycoplasma orale* type 2. In a paper by Fox, Purcell, and Chanock, another oropharyngeal species was identified, and the name *Mycoplasma orale*, type 3, was suggested (9). The fifth of these new species was claimed by Del Guidice, *et al* as a new isolate from a patient with pneumonia (10, 11). The name *Mycoplasma lipophiliae* was suggested because of an affinity for fat stains.

In addition to the above species are an unknown number of "T-strains" of mycoplasma recently described by Shepard (12). These organisms differ from the classical species by the very small colonies produced, and by a requirement for urea. The table presents the species and habitats of mycoplasmas so far reported from man.

MYCOPLASMAS OF HUMAN ORIGIN

Organism	Usual habitat
<i>M. hominis</i>	G. U. Tract, oropharynx
<i>M. salivarium</i>	Buccal cavity
<i>M. fermentans</i>	G. U. Tract
<i>M. pneumoniae</i>	Oropharynx, Bronchi
<i>M. orale</i> , type 1 (<i>M. pharyngis</i>)	Oropharynx
<i>M. orale</i> , type 2	Oropharynx
<i>M. orale</i> , type 3	Oropharynx
<i>M. lipophiliae</i>	Oropharynx
T-strains	G. U. tract, Buccal cavity

Mycoplasma hominis

Habitat: This organism (Fig. 1) is isolated from the genital and rectal mucosa more often than from other regions of the body. It has been isolated on occasions from blood, lungs, and from pus, and occurs in a low percentage of the normal and diseased oropharynges of man. As a tissue culture contaminant, *M. hominis* has been cited as the mycoplasma most frequently encountered (13).

Differentiation: Neither films nor spots are produced on horse serum agar. On horse blood agar, hemolysis is very slight. Good growth occurs on

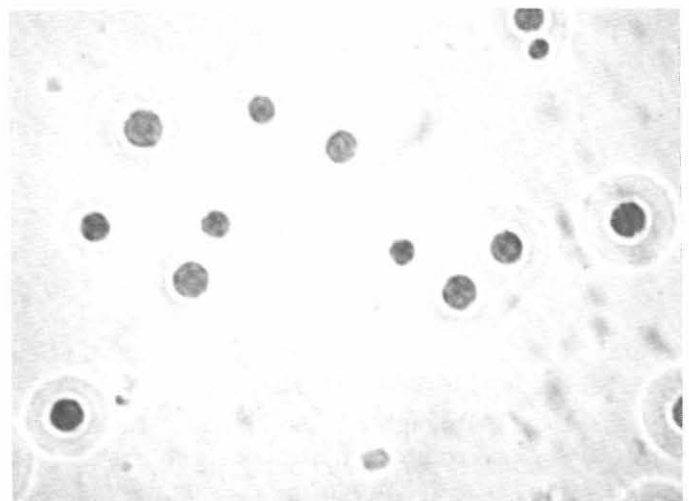


Fig. 1. *Mycoplasma hominis* colonies on the surface of agar medium (X60).

rabbit serum agar, and when semi-solid agar is used the growth is granular throughout the medium. In broth, a very faint opacity is seen with a fine sediment. Carbohydrates are not attacked and reduction of methylene blue is slow and variable. Tetrazolium salts are reduced under anaerobic conditions, and ammonia is produced from arginine. Growth occurs aerobically and anaerobically; however, anaerobic conditions and a pH lower than 7.0–8.0 may favor earlier growth on primary isolation (14). Colonies of *M. hominis* are reported to show smaller cores with broader and rougher peripheral zones than colonies of other species (15). On agar surfaces colonies of *M. hominis* adsorb HeLa cells but do not adsorb spermatozoa or erythrocytes (16).

Pathogenicity: That organisms of this species have a role in nongonococcal urethritis has not been firmly proven. Of 100 mycoplasmas isolated from the genital regions of men and women in Vancouver, B.C., 96 were identified as *M. hominis* (17). An organism thought to be *M. hominis* was isolated from the blood of a patient with a pelvic abscess (18). It has been isolated by several workers from the blood and lungs of mothers and fetuses following abortion (19). Although aerosolized preparations have been reported to produce exudative tonsillitis and pharyngitis in human volunteers, *M. hominis* was not isolated from naturally occurring exudative pharyngitis cases in the U.S. Navy (20) nor from a pediatric population in North Carolina (21). Approximately 1% of young men entering the U.S. Navy harbor *M. hominis* in the oropharynx, while in acute respiratory disease and pneumonia the incidence is only slightly higher. The virulence of *M. hominis* may be in doubt; but the demonstration of subtypes, or antigenic variants occurring with disease processes might be proven if the more sensitive methods of identifying such variants were in use.

Antimicrobials: A variable *in vitro* sensitivity to streptomycin and dihydrostreptomycin has been reported. In the author's laboratory an *M. hominis* strain SDK36, was resistant to gantrisin, sulfathiazole, triple sulfa, penicillin, erythromycin, oleandomycin, polymyxin B, and nalidixic acid (22). It was sensitive to actinomycin D and resistant to thiotepea, velban and 6-mercaptopurine antitumor agents. Sensitivity to optochin (ethylhydrocuprein hydrochloride) has been reported (23).

Significance: Although commonly found in the urethra, and less commonly in the oropharynx, *M. hominis* appears to produce occasional abscesses and blood stream infections. It is an important contaminant of tissue culture lines.

Mycoplasma salivarium

Habitat: This species (Fig. 2) may be isolated from human saliva, tooth scrapings, material from the gingival sulcus, and by cotton swab from tonsillar and pharyngeal regions. Strains also have been recovered from tissues of the African green monkey (24).



Fig. 2. *Mycoplasma salivarium* colonies on the surface of agar medium (X30).

Differentiation: A film and/or spots are usually produced on normal horse serum agar. Hemolysis is not produced on horse blood agar nor under sheep blood agar overlays. Growth on rabbit serum agar is very good. On semi-solid medium there is smooth growth near the bottom. Neither glucose nor other carbohydrates are attacked, but arginine is hydrolyzed with ammonia production. Most strains do best in an atmosphere of 95% nitrogen and 5% carbon dioxide for primary isolation but adapt to aerobic life once growth is well established. *Mycoplasma salivarium* is distinct from other species on the basis of growth inhibition; however, when newly isolated, occasional strains may not be inhibited by specific antisera until many passages have been made.

Pathogenicity: All available information indicates that *M. salivarium* is nonpathogenic. When inoculated experimentally into the nares of gnotobiotic mice, pneumonia was not produced by *M. salivarium* nor by *M. pneumoniae* (25). However, inoculating the rat and mouse lung mycoplasma *M. pulmonis* into these animals did result in gross pneumonia. It is suggested, therefore, that *M. salivarium* pathogenicity, even if existent, may not become evident from animal experimentation.

Antimicrobials: *In vitro* paper disc tests have shown *M. salivarium*, strain PG-21, to be sensitive to neomycin, kanamycin, oxytetracycline, chlortetracycline, novobiocin, chloramphenicol, valsyn, furacin, and to the antitumor compound, actinomycin D. It was resistant to gantrisin, triple sulfa, streptomycin, dihydrostreptomycin, erythromycin, oleandomycin, polymyxin B and nalidixic acid (22).

Significance: As the most common mycoplasma inhabiting the buccal cavity, *M. salivarium* also occurs as a tissue culture contaminant. It has been shown to occur in healthy persons in a straight-line relationship with acute respiratory disease illness rates in a naval recruit population (26).

Mycoplasma fermentans

Habitat: This organism (Fig. 3) is occasionally present on the normal genital mucosa and in ulcerative, genital lesions. It has been isolated in association with fusiform bacilli and spirilla, and may be demonstrated by isolation from either the male or female genital tracts. A strain also has been isolated from the cervix of the African green monkey (24).

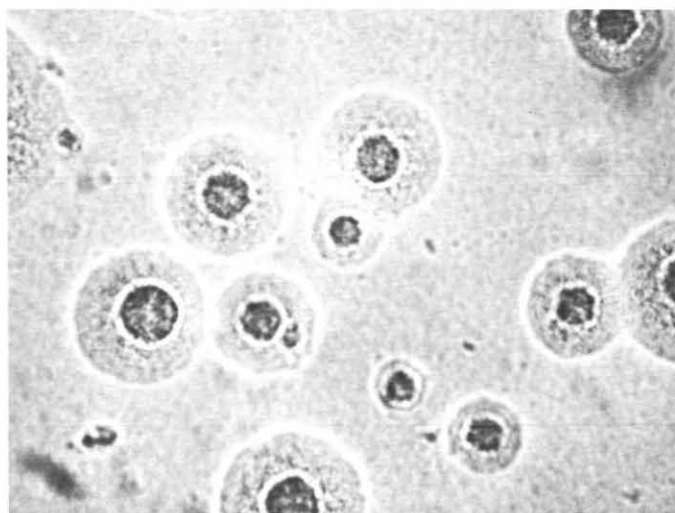


Fig. 3. *Mycoplasma fermentans* colonies on the surface of agar medium. (X55).

Differentiation: A film and/or spots are produced on horse serum agar. No hemolysis occurs on rabbit blood agar, but guinea pig erythrocytes may be lysed by occasional strains. Smooth growth, better near the bottom, is seen in semi-solid medium, and a generalized opacity is produced in broth. Acid is produced from glucose, fructose, galactose, maltose, glycogen, dextrin, and starch; while no acid results from mannose, xylose, sucrose, lactose, salicin, glycerolyn or mannitol. Arginine is hydrolyzed with liberation of ammonia. Reduction of methylene blue

is rapid, and tellurite is reduced anaerobically (23). A few strains have been noted to produce beta-hemolysis under guinea pig erythrocytic overlays (27). Serologically *M. fermentans* is distinct from other species and may be differentiated by the growth inhibition test.

Pathogenicity: *Mycoplasma fermentans* may or may not be pathogenic for mice; abscesses have been reported following foot pad inoculations.

Antimicrobials: *Mycoplasma fermentans*, strain PG-18, was found to be sensitive to streptomycin, tetracycline, oxytetracycline, demethylchlortetracycline, novobiocin, chloramphenicol, valsyn, and furacin (22). Sensitivity was also noted to the following antitumor compounds: thiotepe, velban, and 6-mercaptopurine. All other species of human origin were sensitive to only actinomycin D. Strain PG-18 was resistant to erythromycin, oleandomycin, nalidixic acid, daraprim, and 12 sulfa compounds.

Significance: Role in venereal infections is not clear. Of 100 mycoplasmas isolated from the human urethra, Ford identified only 4 as *M. fermentans* (17). A recent report by Williams, *et al* demonstrated *M. fermentans* in the synovia of a high percentage of rheumatoid arthritis patients (28).

Mycoplasma orale Type 1 (*M. pharyngis*)

Habitat: The pharynx and buccal cavity are the most common sites for isolating *M. orale* type 1. (Fig. 4). Recoveries from bone marrow and peripheral blood have been reported.

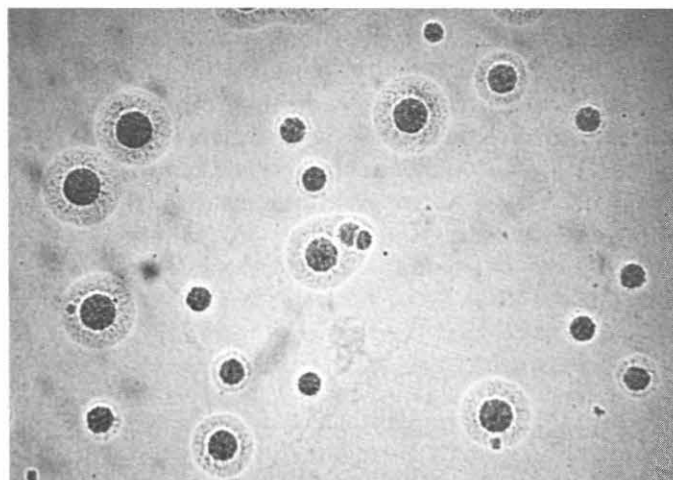


Fig. 4. *Mycoplasma orale* type 1 (pharyngis) colonies on the surface of agar medium. (X70).

Differentiation: Carbohydrates are not fermented. Colonies produce no hemolysis of guinea pig, sheep, horse or human erythrocytes. Arginine is hydrolyzed. Neither film nor spots are produced on horse

serum agar. The colonies often lack surface fringes on primary isolation and may be mistaken for *M. pneumoniae*. By growth inhibition, indirect hemagglutination, and complement fixation, *M. orale* type 1 is clearly distinct from other species although cross reactions with *M. salivarium* rabbit antisera occur in the complement fixation test. While it has been asserted that primary isolation is enhanced if freshly prepared yeast extract is used in the medium, data obtained in the author's laboratory (with yeast autolysate) did not substantiate this claim (29).

Pathogenicity: This mycoplasma is thought to be nonpathogenic for animals or man.

Antimicrobials: A strain of *M. pharyngis*, CD-5, is sensitive *in vitro* to triple sulfa and gantrisin but only in high concentrations (22). This represents the only species tested which is susceptible to these drugs. Strain CD-5 is also sensitive to neomycin, kanamycin, oxytetracycline, chlortetracycline, novobiocin, chloramphenicol, polymyxin B (high conc.) nalidixic acid, valsyn, furacin, actinomycin D, bryamycin, and daraprim. Sensitivity to optochin has been reported (23). It is resistant to streptomycin, dihydrostreptomycin, erythromycin, and oleandomycin.

Significance: The presence of *M. orale* type 1 in the human pharynx has not been correlated with acute respiratory disease symptoms; however, a poorly understood linear relationship of isolations with acute respiratory disease illness rates has been demonstrated in naval recruits (26). The recovery of *M. orale* type 1 and other mycoplasmas from clinical specimens taken from leukemia and tumor patients prompted more extensive research into the role of mycoplasmas in these conditions; however, the results of such studies have not confirmed such a role (30). *Mycoplasma orale* type 1 is a frequent contaminant of tissue cultures and is known to suppress Rous Sarcoma virus and to produce cell destruction of human diploid and chick-embryo fibroblasts (31).

Mycoplasma orale Type 2

Habitat: This organism (Fig. 5) has been isolated from the human oropharynx; once from the oropharynx of a child and 3 times from the oropharynges of adults (8). It has also been recovered from the pharynges, cervixes and prostatic urethrae of African green monkeys (24).

Differentiation: *Mycoplasma orale* type 2 produces fried egg colonies on PPLO agar, and when overlaid with guinea pig erythrocytes alpha-hemolysis is

produced around the colonies. Glucose is not fermented and arginine is utilized with an upward pH shift. Tellurite is reduced aerobically and anaerobically. A strain (ATCC23204) was the only species of human origin found to be positive for phosphatase (23). Complement fixation shows differences from other species but some relation to *M. pharyngis* and *M. arthritidis*. Other serological tests, i.e., gel diffusion and indirect hemagglutination, are more specific in their reactions than the complement fixation test. By growth inhibition, *M. orale* type 2 is distinct from all other recognized species of human origin.

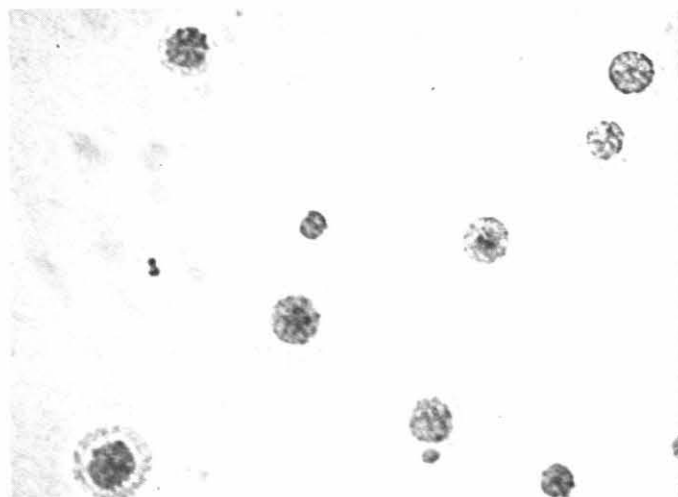


Fig. 5. *Mycoplasma orale* type 2 colonies on the surface of agar medium (X70).

Pathogenicity: Experimental data or clinical information is not available on this organism's role in disease.

Antimicrobials: Information on antibiotic sensitivities is not available. Aluolto *et al* found that a strain was sensitive to optochin (23).

Significance: *Mycoplasma orale* type 2 is reported infrequently. Taylor-Robinson *et al* obtained 4 isolates (8), and Clyde reported 2 isolates (32). *Mycoplasma orale* type 2 has not been identified among the isolates from naval recruits (33).

Mycoplasma Orale Type 3

Habitat: Six isolates so far reported were all recovered from the human oropharynx of adult patients (9). A strain has been isolated from the pharynx of an African green monkey (24).

Differentiation: Fried egg colonies are produced on PPLO agar and are weakly hemolytic when covered with guinea pig erythrocytic overlays. Arginine is attacked with a rise in pH while glucose and urea are not utilized. Growth is inhibited by 0.001%

methylene blue, and 2, 3, 5-triphenyl-2H-tetrazolium chloride is not reduced under aerobic conditions. Fresh yeast extract is required for growth. Occasional strains have shown a one-way relationship with *M. pharyngis* and *M. salivarium* by complement fixation tests.

Pathogenicity: No information available.

Antimicrobials: No information available.

Significance: This organism has accounted for 1.4% of 437 mycoplasma isolates. *Mycoplasma orale* type 3 may be considered a rare member of the normal mycoplasma flora.

Mycoplasma lipophilae

Habitat: One isolate has been reported from sputum of a pneumonia patient (10).

Differentiation: On medium containing Oil Red O stain, *M. lipophilae* colonies are bright red (11). Fluorescent antibody and growth inhibition tests indicate that this strain is serologically distinct from other species of human origin.

Pathogenicity: No information available.

Antimicrobials: No information available.

Significance: Whether or not this organism is of sufficient importance to be considered in the listing of mycoplasmas stemming from man is doubtful, i.e., only 1 isolate is reported.

Mycoplasma pneumoniae (Eaton Agent)

Habitat: This species (Fig. 6) may be recovered from the oropharynx, sputum and bronchial secretions.

Differentiation: Dextrose, xylose, mannose, maltose, dextrin, and starch are utilized with the formation of acid. A peroxide hemolysin completely destroys guinea pig and sheep erythrocytes producing zones of beta-hemolysis around the colonies. The reduction of 2, 3, 5-triphenyl-2H-tetrazolium chloride occurs under aerobic conditions. Tellurite is reduced aerobically (23). The colonies have finely granular surfaces, more often lack peripheral surface zones, and present a "mulberry" appearance with a golden hue. Whereas other species are inhibited by methylene blue chloride in agar medium, *M. pneumoniae* grows well in the presence of this dye. Methylene blue is reduced anaerobically. A smooth haze of turbidity is produced in fluid medium and microcolonies or aggregates of organisms (microcolonies) are often seen in hanging drops. The capacity of hemadsorbing certain mammalian erythrocytes is demonstrated by covering colonies of *M. pneumoniae* with appropriate cell suspensions (34).

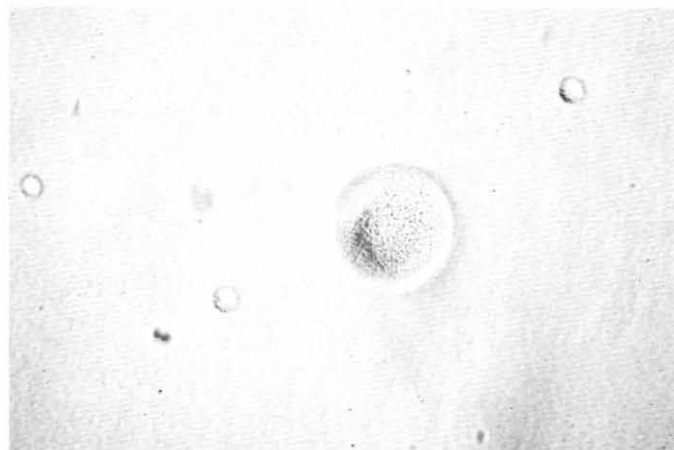


Fig. 6. *Mycoplasma pneumoniae* colonies on the surface of agar medium. (X100).

Pathogenicity: Growth occurs on the bronchial epithelia of embryonated chick eggs, in primary chick embryo, guinea pig, monkey, and human cell tissue cultures. Hamsters and cotton rats, which are susceptible to infection, usually develop lung lesions in 10 to 14 days. In contrast, gnotobiotic mice inoculated with *M. pneumoniae* do not develop lung lesions. In human volunteers experimentally infected with *M. pneumoniae*, inapparent infections, acute respiratory disease, and pneumonia have been reported (35). Strains maintained in agar cultures lose virulence, whereas freshly isolated strains or those maintained in tissue culture retain their virulence. Infected sections of hamster trachea, planted in organ cultures and studied by immunofluorescence, show paralysis of cilia, inter- and paracellular attachment of *M. pneumoniae* but no evidence of intracellular invasion (36).

Antimicrobials: *Mycoplasma pneumoniae* is sensitive *in vitro* to tetracyclines, erythromycin, streptomycin, dihydrostreptomycin, novobiocin, chloramphenicol, oleandomycin, polymyxin B (high conc.) valsyn, faracin, actinomycin D, bryamycin, daraprim and tylosin tartrate. It is also sensitive to optochin (23). It is resistant to 12 sulfa compounds, neomycin, kanamycin, and nalidixic acid. Erythromycin and tetracycline have been used therapeutically in the treatment of *M. pneumoniae* pneumonia (37). In Japan, an erythromycin-resistant strain has emerged (38).

Significance: This species is the only member of the genus whose pathogenicity for man is firmly established. In military recruits and college students in the south, infection has been demonstrated in 41-44.3% of large samplings of primary atypical pneumonia cases (39). *Mycoplasma pneumoniae* is also an important cause of pneumonia in non-

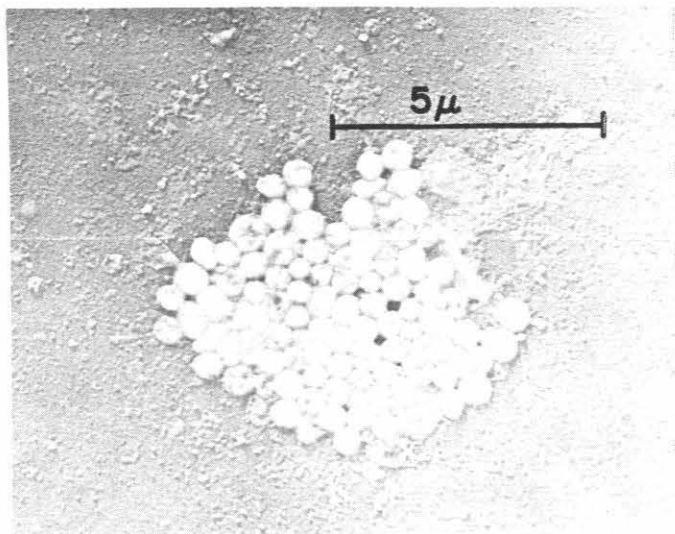


Fig. 7. Colonies of T-strain mycoplasma No. 960 (from M. C. Shepard) growing on the surface of agar medium. Reproduction with permission of Dr. W. A. Clyde, Jr. and the editor of "Annals of the New York Academy of Sciences."

student civilian populations (40). In addition to relevance in pneumonia, this organism has been reported in cases of Stevens-Johnson syndrome (erythema multiforme exudativum) (41). These studies were based on antibody measurements or were presented as case histories on single individuals. In the author's laboratory, only 2 infections in 30 cases did not confirm *M. pneumoniae* as an important factor in this syndrome (42). More recently *M. pneumoniae* infections have been reported in children suffering with the Guillain-Barré syndrome, a rare neurological disorder that follows an upper respiratory infection (43). For the future, the possibility exists that this organism may appear in other disorders that, presently, are of unknown etiology. A death due to *M. pneumoniae* infection has been documented (44).

T-strains

Habitat: T-strains (T=tiny form colonies) (Fig. 7) are isolated from the genitourinary tract and the oropharynx.

Differentiation: The biological properties of T-strain mycoplasmas have been thoroughly described by Shepard (12). Very small colonies ranging in diameter from 7 to 24 μ , but not exceeding 70 μ , are produced on special media. Surface zones at the periphery of the colony are seen but rarely. Growth occurs in several special media from which thallium acetate must be omitted. Fluid cultures develop no evidence of turbidity. The optimal pH for growth

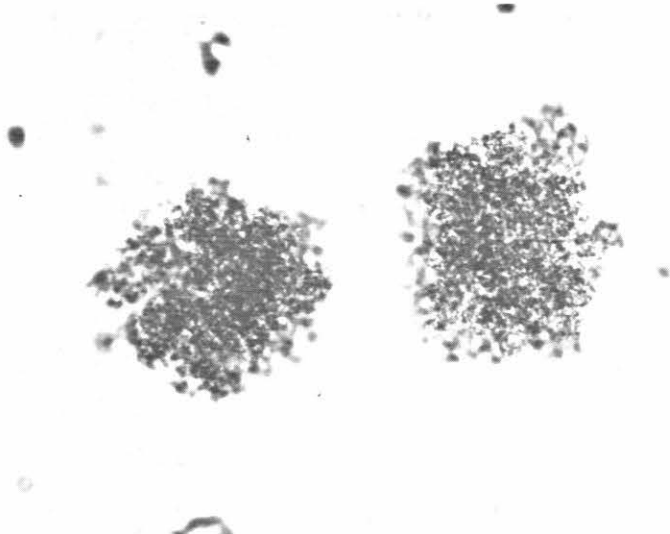


Fig. 8. A colony of the Boston T-strain of mycoplasma (X533). Reproduction with permission of Dr. R. B. Kundsins and the editors of "Science."

in artificial medium is 6.0. Carbohydrates are not fermented. Urea is hydrolyzed with the production of ammonia which is toxic. T-strains are catalase negative.

Pathogenicity: Information is not available on pathogenicity for animals. Indirect evidence, cultural and histological studies are consistent with the hypothesis that T-strains may act as pathogens in certain genitourinary conditions.

Antimicrobials: The T-strains of human genital origin are sensitive *in vitro* to tetracycline, oxytetracycline, erythromycin and 5-iodo-2'-deoxyuridine. It is of interest that these compounds have been used effectively in the treatment of nongonococcal urethritis (45).

Significance: First demonstrated in nongonococcal urethritis by Shepard in 1954, the T-strains have been found more frequently in this condition than classical large colony mycoplasmas. Kundsins, Driscoll and Ming isolated a T-strain from the chorion, decidua, and amnion of a patient who sustained a spontaneous abortion (46). Designated the Boston T-strain (Fig. 8), it differed from other T-strains by a slower rate of growth and by its demand for more exacting nutritional requirements. More recently, T-strains were isolated from 85% of females with genitoinfectious disease whereas *Neisseria gonorrhoeae* was recovered from only 24% (47). These data suggest that if T-strain mycoplasmas are confirmed as true etiological agents of genitoinfectious disease they are a greater problem than the gonococcus.

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STOCK SOLUTIONS AND REAGENTS

Before preparing media, attempting isolations, or beginning serological procedures, both time and inconvenience will be spared if the following solutions and reagents are prepared and stored in advance. The source of material will be found in the supply table.

1. *Fresh, aqueous yeast extract.* The extraction is made from fresh baker's yeast commercially available as "Fleischmann's Pure Dry Yeast Type 20-40" listed in the table of supplies. Little is known of the merits of other yeasts, although it is known that some are active. Yeast extracts or autolysates in the dehydrated form which are adequate for most species are deficient for the primary isolation of *M. pneumoniae*. Fresh aqueous yeast extracts similar in composition to the below preparation are also available from various commercial sources (see supply table).

FORMULA FOR 1 LITER

Baker's yeast	250 g
Distilled water	1 liter

Add the yeast to the water and bring the mixture to a boil. Hold at boiling at least 2 min. An alternate procedure is to heat in an autoclave 5 lb. for 10 min. Allow to cool and filter by gravity through 2 layers of filter paper of medium porosity, such as Whatman No. 12. Usually, 300 to 350 ml will successfully pass the filter. The pH of the slightly turbid filtrate ranges from 6.4-6.6 and must be adjusted to pH 7.8-8.0 with 1 N NaOH. Note: For T-strains, set pH at 6.0 with HCl. Ten ml quantities of extract are placed in screw-cap tubes and sterilized at 121 C for 15 min in the autoclave. Storage is in the frozen state at -20 C. Before incorporating into the medium, the thawed extract should be clarified by centrifugation at 1500-2000 rpm in an angle-head, table-top centrifuge. Only the clear supernatant solution is decanted into the medium.

2. *Kenny's yeast dialysate.* A yeast dialysate enrichment instead of the crude extract has been used by Kenny (1). The advantages of his preparation are those of purity and clarity, while improved specificity and more sensitive serological reactions may be ex-

pected. "Fleischmann's Pure Dry Yeast Type 20-40" is again the yeast of choice.

FORMULA

Baker's yeast	980 g (2 lb)
Distilled water	2,500 ml

Heat yeast suspension in autoclave at 5 lb for 10 min and allow to cool. Place filtrate in dialysis casings and dialyze against 2.0 L water for 48 hr at 4 C. Save dialysate (fluid outside casing). Adjust pH, dispense 10 ml quantities in screw-cap test tubes and sterilize by autoclave at 15 lb for 15 min. Store frozen at -20 C.

3. *Amphotericin B (Fungizone).* This antimicrobial is used to inhibit certain contaminating fungi which grow abundantly on mycoplasma agar medium. It is especially necessary if media are to be incubated in the humid atmosphere of anaerobic jars. To each 50 mg of the powder, add 10 ml sterile distilled water. Placing the container on a Kahn shaker or other shaking device for 5-10 min hastens solution of the material. Store at 4 C. Activity in media may decline rapidly after 1 week.
4. *Penicillin in horse serum supplement.* Penicillin is incorporated in mycoplasma medium as a routine measure to suppress Gram-positive bacteria. It may be conveniently added to the final medium by way of the horse serum enrichment. Using a 5.0 ml syringe and 21-g needle remove 5.0 ml from a 100 ml horse serum bottle and inject into a vial containing 1,000,000 units penicillin G. When fully dissolved transfer the 5.0 ml back into the horse serum bottle. Store at 4 C. The concentration of penicillin in media containing 20% horse serum will be 2,000 units/ml; in media containing 10% horse serum the concentration will be 1,000 units/ml.
5. *Tetrazolium solution.* This reagent may be used for the biochemical identification of *M. pneumoniae* in the tetrazolium color test. Prepare a 1.0% solution as follows: Dissolve 1.0 g of 2,3,5-triphenyl-2H-tetrazolium chloride in 100 ml of distilled water. Transfer solution to screw-cap bottle and sterilize in an autoclave at 121 C for 15 min. The solution is stable for as long as 12 months at 4 C.

STOCK SOLUTIONS AND REAGENTS

6. *Methylene blue chloride*. This solution is used for incorporation in selective medium for the isolation of *M. pneumoniae* (2). A 1.0% solution is made by dissolving 0.1 g of the dye in enough distilled water for 10.0 ml of final solution. It may be sterilized by autoclave at 121 C for 15 min and stored at 4 C in a screw-cap tube.
7. *Dienes' stain*. This solution is used for the microscopic study of colonies. Also available commercially, the Dienes' formula for cover-slip staining is as follows (3,4).

FORMULA FOR 20 ML. DIENES' AQUEOUS STAIN

Methylene blue chloride	0.50 g
Azure II	0.25 g
Maltose	2.00 g
Sodium carbonate	0.05 g
Benzoic acid	0.04 g
Water	20.00 ml

Using a cotton applicator, or loop, smear a thin film of stain over the surface of clean coverslips and allow to dry. A loopful of 0.05% phosphoric acid may be smeared over the dried stain and allowed to evaporate. The added phosphoric acid is thought to improve the staining reaction; however, good results are obtained without phosphoric acid. The stained coverslip is conveniently stored in a petri dish stained side down. An alcoholic solution of comparable reliability spreads more easily and dries more rapidly. It is prepared by dissolving 0.50 g methylene blue chloride and 0.25 g Azure II in 20 ml 95% ethyl alcohol.

8. *Thallium acetate*. This solution is used for incorporation in small volumes of media. Thallium acetate, 0.5 mg/ml is used in mycoplasma media to suppress the growth of Gram-negative bacteria. Because it is difficult to weigh the crystals for small volumes of medium, a 10% solution is more conveniently handled.

FORMULA FOR 100 ML 10% THALLIUM ACETATE SOLUTION

Thallium acetate	10 g
Distilled water, q. s.	100 ml

The volume used is 0.5 ml in each 100 ml medium.

9. *Phenol Red*. Prepare 2.0% and 0.5% solutions of phenol red for use as color indicator of pH change in dextrose fermentation, arginine, and urea utilization reactions.

FORMULA FOR 100 ML PHENOL RED SOLUTIONS

	0.5% solution	2.0% solution
Phenol red	0.5g	2.0 g
Water, q. s.	100.0 ml	100.0 ml

10. *Sterile 1 N HCl*. Prepare a sterile 1 N HCl solution for aseptic adjustment of pH when adding horse serum to T-strain medium. Previously standardized 1 N HCl solution is sterilized in the autoclave at 121 C for 15 min.
11. *Paraffin*. Prepare a pot of paraffin for sealing plates. An airtight seal is easily made by adding melted paraffin around the lid of the Petri dish. Place approximately one-fourth lb paraffin in a beaker and melt in a boiling water bath. *Do not place beaker directly over burner*. An automatic electric paraffin melter operating at 92 C is more convenient for this purpose.
12. *Paraffin mixture*. Prepare a mixture of 90% paraffin and 10% vaseline. Melt as in No. 11 above, mix, and save to make agar mounts for microscopic examinations.
13. *Phenolized veronal buffer*. This buffer is used for *M. pneumoniae* complement fixing antigen.*

FORMULAE

Stock Solution A

Diethyl barbituric acid	4.60 g
Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	0.20 g
Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	1.00 g
Dissolve in 500 ml distilled water at 90 C	
Cool before adding to stock solution B.	

Stock Solution B

Sodium chloride	83.80 g
Sodium bicarbonate	2.52 g
Sodium diethyl barbiturate	3.00 g
Dissolve in 500 ml distilled water.	

Stock Solution C

Mix A with B and bring the resulting 1 liter volume to 2 liters with distilled water; pH should be 7.3 to 7.5. This solution is hypertonic and requires further dilution.

Working Solution D

Dilute 1 part C with 4 parts distilled water and mix well. Then to 99.5 ml of this mixture, add 0.5 ml liquid phenol and mix.

14. *Alsever's solution*. This solution is used for preservation of erythrocytes for use in the hemolytic plaque test.

* Veronal buffered saline *without* phenol is used as diluent in all CF testing except antigen preparation.

FORMULA FOR 1 LITER

Anhydrous dextrose	18.66 g
Sodium chloride	4.18 g
Sodium citrate	8.00 g
Citric acid	0.55 g
Distilled water, q.s.	1 liter

When dissolved, sterilize in an autoclave at 10 lb pressure for 10 min. Freshly drawn whole blood is thoroughly mixed with an equal volume of the sterile Alsever's solution. This mixture can be stored at 4 C for a month or longer.

15. *Phosphate buffered saline.* These solutions have been used for washing mycoplasma organisms such as *M. pneumoniae* growing on glass surfaces. Isotonic phosphate buffered solutions of various pH reactions may be prepared from 3 stock solutions (5).

Step 1. Prepare 3 stock solutions as follows:

- A. Stock solution NaCl (0.1540 M)
Formula for 1 Liter
NaCl 9.00 g
Water, q.s. 1 liter
- B. Stock solution KH_2PO_4 (0.1540 M)
 KH_2PO_4 20.96 g
Water, q.s. 1 liter
- C. Stock solution Na_2HPO_4 (0.1027 M)
 Na_2HPO_4 14.59 g
Water, q.s. 1 liter

Step 2. Mix as follows for one liter of working solution of desired pH reaction:

Final pH	ml NaCl Solution A	ml KH_2PO_4 Solution B	ml Na_2HPO_4 Solution C
6.4	500	324.5	175.5
6.6	500	263.0	237.0
6.8	500	200.0	300.0
7.0	500	149.0	351.0
7.2	500	102.5	397.5
7.4	500	62.5	431.5
7.6	500	45.0	455.0
7.8	500	29.0	471.0
8.0	500	18.5	481.5

16. *Pooled normal guinea pig serum.* Prepare pooled serum of normal guinea pigs for use in antimetabolic serological tests if such work is contemplated. Normal guinea pig serum in concentrations of 2.5–5% are added to diluent because it has been shown that metabolic serum titers are enhanced. Fresh normal sera are collected from guinea pigs and pooled. One and 5.0 ml quantities are then stored at –20 C in sealed glass ampules. This serum is inactivated undiluted at 56 C for 30 min just before adding to the diluting medium used for running the test (page 46).

17. *Pooled rabbit serum.* Prepare pooled rabbit serum for use as diluent in the indirect hemagglutination test. Bleed rabbits, collect sera, pool and inactivate at 56 C for 30 min. To each ml of serum add 0.5 ml of washed sheep erythrocytes. Allow adsorption to take place at 4 C for 30 min. The serum is then separated from the red blood cells by centrifugation and stored frozen at –20 C.

18. *Hemolysin.* Prepare a 1:100 stock solution of hemolysin for complement fixation tests.

Veronal buffered saline	94.0 ml
Phenol (5% in saline)	4.0 ml
Glycerinized hemolysin	2.0 ml

Add the phenol to the saline and mix well before adding hemolysin. Store at 4 C.

19. *Sheep erythrocyte suspension.* Prepare a 2% suspension of sheep erythrocytes for complement fixation tests. To each volume of sheep blood add 3 volumes of cold veronal buffered saline and spin in an angle head bench top centrifuge at 2,000 rpm for 5 min. Discard the supernatant and wash the sediment 3 times with saline. Subject the 3rd washing to 10 min of spinning to pack the red blood cells. To each 0.1 ml of packed cells add 4.9 ml saline for obtaining 2% suspension.

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MEDIA

Standard Medium for Isolation and Propagation of *M. pneumoniae* and Other Classical Species

For the isolation and propagation of *M. pneumoniae* and other species of human origin, the PPLO agar and PPLO broth of Morton, *et al* as modified by Hayflick, is presently the medium of choice (1,2). The agar medium is composed of 70% PPLO agar, 20% horse serum, and 10% of aqueous 25% yeast extract. Inhibitors are included as follows: penicillin, 2000 units/ml; thallium acetate, 0.5 g/L; and amphotericin B, 5.0 μ g/ml (see Fig. 9).

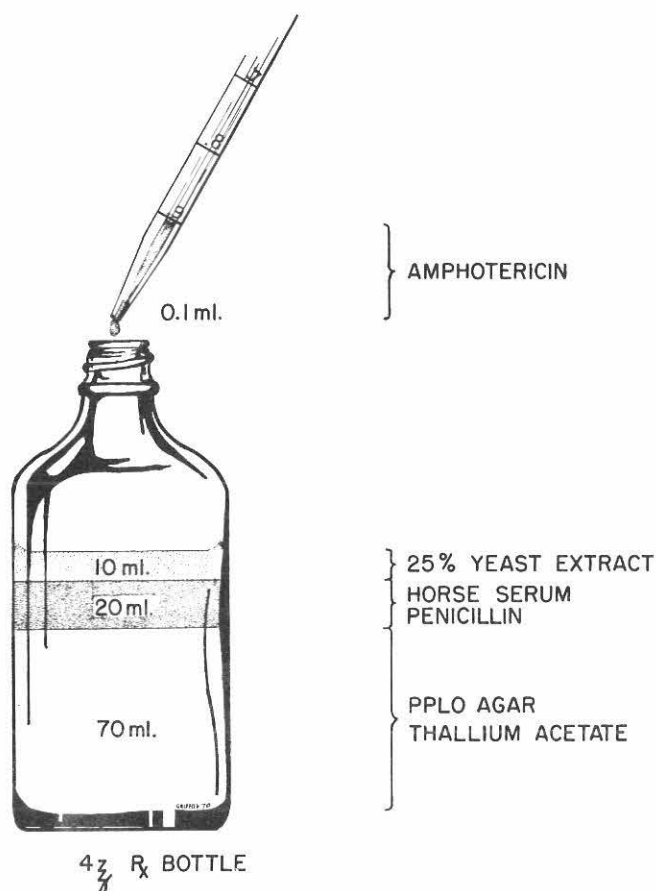


Fig. 9. Preparation of 100 ml bottle of "standard" PPLO agar for isolation and preparation of *M. pneumoniae* and other species.

STEP 1

Distilled Water	1 liter
PPLO agar	35.0 g
Thallium acetate	0.5 g

Heat to boiling or until dissolved. Adjust pH to 7.8–8.0 with 1 N NaOH. Seventy ml portions are then dispensed in 4-fluid-ounce prescription bottles

and sterilized at 121 C for 15 min. When cooled to room temperature, the caps are tightened, and the bottles stored at room temperature until needed. When supplemented, one bottle provides enough medium for pouring 14 to 17 (60×20 mm) plastic plates—0.5 cm thick. One bottle of medium is sufficient for 5 to 7 (100×15 mm) plates.

STEP 2

Melt the agar from step 1 by placing the bottles in an autoclave or in a boiling water bath after first loosening the caps. Cool to 56 C, then add 10 ml of 25% yeast extract, 20 ml horse serum-penicillin solution, and 0.10 ml stock solution amphotericin B. Mix and pour 7.0 ml for a small 60×20 mm petri dish and 15 ml for a standard 100×15 mm dish.

Medium with Methylene Blue Chloride for Selective Isolation of *M. pneumoniae*

The medium described supports the growth of *M. pneumoniae* and other species as well. This fact is of concern because more rapidly growing species may mask the presence of *M. pneumoniae* in the culture. Also, more time and effort will be required to separate and identify the colonies that appear on this medium when only *M. pneumoniae* is under study. It has been shown that incorporating 0.002% methylene blue chloride to standard *M. pneumoniae* agar prevents growth of other species, yet allows *M. pneumoniae* to multiply into normal colonies (3). In order to achieve the selective action, and to exclude *M. hominis* I it is important to incubate aerobically and to adjust the pH to 7.8–8.0. Some laboratory adapted strains of *M. hominis* I will grow in the presence of 0.002% methylene blue if incubation is aerobic and the pH is 7.2 and lower. Many *M. hominis* I strains will also grow in the presence of methylene blue under anaerobic conditions, but not at pH 7.8.

From the 1.0% stock solution of methylene blue, add 0.20 ml to 100.0 ml melted agar medium just before pouring the plates. The final agar is pale blue to greenish in hue.

Diphasic Medium for Isolation of *M. Pneumoniae* and Other Species

It has been long asserted that a layer of agar covered with fluid medium improves the growth of mycoplasmas. Advantage of this principle has been taken by Kenny, who recommends such a system for

the isolation of *M. pneumoniae* from clinical material (4,5). When this medium is incubated in an aerobic atmosphere without CO₂, it is still relatively selective for *M. pneumoniae* and *M. hominis*. However, when incubated in 95% N₂-5% CO₂, the agar medium readily isolates *M. pharyngis* and *M. salivarium*. In an extensive survey of Mycoplasmataceae, Kenny found that only T-strains could not be readily cultivated. Nevertheless, the diphasic medium described below was originally developed and tested for the isolation of *M. pneumoniae*. The diphasic medium of Kenny is prepared as follows:

BASAL AGAR MEDIUM	
Soy peptone	20 g
NaCl	5 g
Agar	10 g
Water	1 liter

Adjust pH to 7.4 with 1 N NaOH. Sterilize in an autoclave at 121 C for 15 min. Store at room temperature.

BASAL FLUID MEDIUM	
Soy peptone	20 g
NaCl	5 g
Glucose	10 g
Water	1 liter
2% phenol red	2 ml

Adjust pH to 7.6 with 1 N NaOH and sterilize.

COMPLETE SOLID MEDIUM FOR PLATING AND TUBING

Basal agar medium	65%
Horse serum	25%
Yeast dialysate	10%

(see p. 11)

Thallium acetate 1:3000 final concentration	
Penicillin	200 units per ml

COMPLETE FLUID MEDIUM CONTAINS SAME ENRICHMENTS AS COMPLETE SOLID MEDIUM ABOVE

Diphasic medium is prepared by allowing 2-3 ml of complete solid medium to harden in a screw-cap tube. Then 3-4 ml complete fluid medium is added. The caps should be turned tightly to prevent excessive alkalinity (over pH 7.8).

The tubes are inoculated with the clinical specimen and incubated at 36-37 C for 43 days. As the pH drops a color change occurs. Subcultures to agar plates are then made. Routinely, subcultures are made at 5, 21 and 42 days on solid medium. The diphasic medium is stored frozen on the 42nd day.

SPECIMEN COLLECTION

Throat swabs are suspended in 2-5 ml Trypticase Soy Broth containing 0.5% bovine serum albumin. Media are inoculated with 0.1 ml of this material.

Note: An alternate diphasic medium containing phenol red and methylene blue has been described by Smith, *et al* (6).

Medium for Isolation and Propagation of Mycoplasmas Other than *M. pneumoniae*

While the isolation of *M. pneumoniae* is customarily carried out under aerobic conditions, most other species require anaerobic or microaerophilic incubation on primary isolation. The exceptions that occur are rare events in any given series of cultures. The recovery from the respiratory tract of *M. salivarium*, *M. pharyngis* and *M. hominis* to the virtual exclusion of *M. pneumoniae*, can be achieved on a medium more economically prepared than the hyperenriched medium needed for *M. pneumoniae* (7, 8). This medium has been used by Shklair and associates who reported mycoplasmas from 87.5% of human saliva specimens (9). In extensive comparative studies the following agar medium was found equivalent to the standard medium for isolating oropharyngeal species other than *M. pneumoniae* (10).

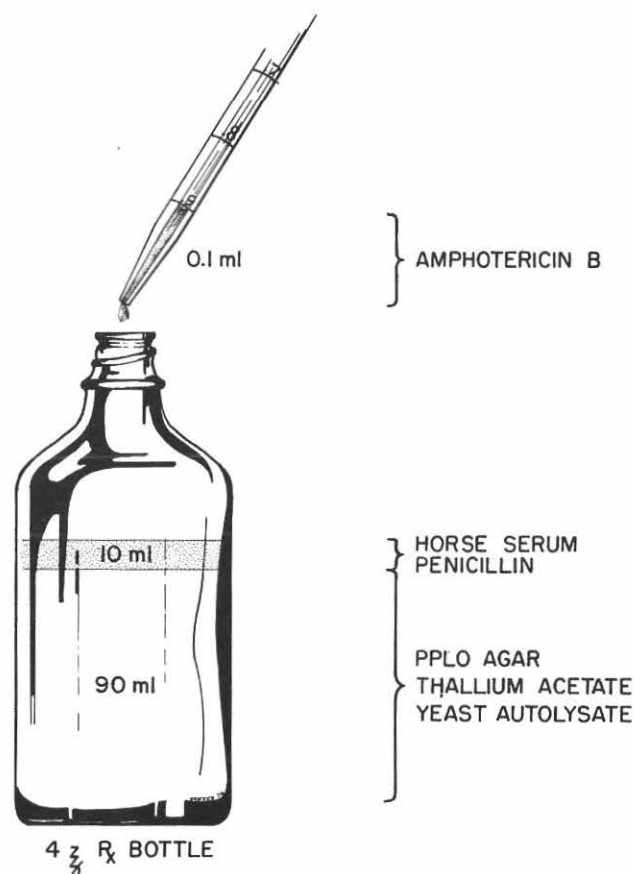


Fig. 10. Preparation of 100 ml bottle of PPLO agar medium for isolation and preparation of *M. salivarium*, *M. hominis*, and *M. orale* type 1.

MEDIA

FORMULA

Distilled water	900 ml
PPLO agar	35.0 g
Thallium acetate	0.5 g
Yeast autolysate*	10.0 g

Heat to boiling or until all ingredients are dissolved. Lower pH to 6.0 to 7.0 with 1N HCl (11). Ninety ml volumes are then dispensed in 4-fluid-ounce prescription bottles and sterilized at 121 C for 15 min. Storage is at room temperature. Before pouring, the medium is melted, cooled to 50–56 C, and the following additives incorporated:

Horse serum-penicillin	10 ml
Amphotericin B	0.1 ml

The plates are poured in the usual manner. Incubation of the *unsealed* plates is carried out at 37 C in anaerobic jars evacuated and flushed 3 consecutive times with a mixture of 95% N₂—5% CO₂ gases (see page 22).

* *Comment on Yeast Autolysates:* The water solubility and the ability of the commercially available yeast autolysates to support growth of mycoplasmas is highly variable. The recommended autolysate, "Basamin Busch," formerly prepared by Anheuser-Busch, Inc., St. Louis, is no longer available. The brand recommended in the table of supplies was found to be equivalent to the Anheuser-Busch product and to be superior to certain other brands on the market for (a) water solubility and (b) the growth of mycoplasma (8).

Fluid Medium for Dextrose Utilization by *M. pneumoniae* and *M. fermentans**

The breakdown of dextrose oxidatively by *M. pneumoniae*, and fermentatively by *M. fermentans* may be detected areobically by using broth medium of the following composition:

FORMULA FOR 1 LITER

Water	1 liter
PPLO Broth	21.0 g
Thallium acetate	0.5 g
Dextrose	14.0 g
Phenol red, 2%	1.4 ml
pH 7.8	

Heat to dissolve all ingredients. Transfer 3.5 ml into 16 × 125 mm screw-cap test tubes or other tubes of suitable volume. Sterilize by autoclaving at 121 C for 15 min with caps loosened. When cooled, add to each tube 1.0 ml horse serum-penicillin solution, and 0.5 ml 25% fresh yeast extract previously sterilized. Final concentration of dextrose is 1.0%. An alter-

* When preparing liquid medium, "PPLO Broth" is substituted for "PPLO Agar". The enrichment is the same as for solid medium except that amphotericin B is omitted because fungal contamination of fluid medium is rarely encountered.

nate method is to add a sterile solution of dextrose separately after cooling from the autoclave. Sterile solutions of 10% dextrose are commercially available (see supply list). Another medium has been described for detecting breakdown of dextrose (12).

Fluid Medium for Arginine Utilization by Mycoplasmas Other Than *M. pneumoniae*

The degradation of arginine by species other than *M. pneumoniae* may be detected by upward pH shift and resulting color change of the following medium:

FORMULA FOR 1 LITER BASAL FLUID MEDIUM

Water	1 liter
PPLO Broth	21.0 g
Thallium acetate	0.5 g
Phenol red, 2%	1.4 ml
pH 7.0	

Heat to dissolve all ingredients. Prepare 10% (w/v) stock solution L-arginine hydrochloride by dissolving in an aliquot removed from above basal medium and sterilize by filtration. Save for addition to complete fluid medium. Add 3.0 ml basal medium to 16 × 125 mm screw-cap test tubes and sterilize by autoclaving at 121 C for 15 min with caps loosened. When cooled add 1.0 ml horse serum-penicillin solution, 0.5 ml yeast extract adjusted to pH 7.0, and 0.5 ml arginine-medium solution previously sterilized by filtration. Final concentration of arginine is 1.0%. Final pH should be 7.0–7.2. Another medium has been described (12).

Special Fluid Medium for Cultivating *M. pneumoniae* On Glass Surfaces for Use in Immunodiffusion Reactions and Complement Fixation Tests

A buffered medium has been described by Conant, *et al*, who recommended that it be used for cultivating *M. pneumoniae* on glass surfaces (13). The buffer prevents the rapid drop in pH which alters the antigenicity of *M. pneumoniae*. This medium is supplemented with PPLO serum fraction and Eagle's Minimum Essential Medium with horse serum omitted. The liquid form of Eagle's Minimum Essential Medium has been here substituted for the powdered form to avoid the problem of insolubility. Although this special medium was devised for preparing antigens for immunodiffusion reactions, it also yields superior harvests of *M. pneumoniae* complement fixation antigen. The special fluid medium of Conant, *et al*, slightly modified, is compounded as follows:

FORMULA FOR 1 LITER

Water	794.5 ml
PPLO Broth, Difco	7.4 g
N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)	11.9 g
Thallium acetate solution, 10%	5.0 ml
Phenol red solution, 2%	0.5 ml
pH 7.4	

Sterilize by autoclave 121 C 15 min. When cooled to room temperature, add the following enrichments and antibiotics:

PPLO serum fraction with 500,000 units penicillin	50.0 ml
Fresh yeast extract, aqueous	50.0 ml
Eagle's Minimum Essential Medium* (10X)	50.0 ml
Glucose, filtered, 10%**	50.0 ml

Final pH 7.4

* Eagle's Minimum Essential Medium contains Earls' salts without sodium bicarbonate and with L-Glutamine.

** Glucose may be added at 5.0 g to the formula to be autoclaved; in this case add 50.0 ml H₂O or an initial 844.5 ml.

Broth Medium for Preparation of *M. pneumoniae* Hemagglutinin (Feldman-Suhs Modification)

The preparation of hemagglutinating antigen from *M. pneumoniae* has been explored by Feldman and Suhs (14, 15). It contains dextrose, and alkaline cresol red indicator. The cresol red indicator is substituted for phenol red because the color changes at a higher pH, i.e., 7.1 to 7.2. This permits transfers to be made before the culture becomes overly acidified. The formula of this broth medium is as follows:

PPLO Broth, (Difco)	70.0 ml
Horse serum, GG-free*	20.0 ml
Yeast extract (20-40, 25%)	10.0 ml
Dextrose (20%)	5.0 ml
Penicillin G (50,000 u)	1.0 ml
Thallium acetate (10%)	0.5 ml
Alkaline cresol red (0.4%)	0.1 ml
pH 7.8	

It may be noted that the final volume is 107.5 instead of the usual 100 ml. Horse serum and penicillin are added separately.

Urease Color Test Medium U9 for Detection of T-Strain Mycoplasmas

T-strain Mycoplasmas possess a urease system whereas other classical species have no urease

* *Important Note on Horse Serum:* Pooled normal horse serum contains agglutinins for erythrocytes of vervet monkeys, rabbits, and humans. Hence the horse serum used for preparing hemagglutinin medium must be free of gamma globulin.

(16, 17). The incorporation of urea and phenol red provides a system of color change. As urea is utilized and ammonia accumulates, the pH rises, and the medium turns from yellow to pink or red. An uninoculated control tube is incubated together with the specimen tube. A positive reaction is generally obtained in the test vial in 18-24 hr and usually begins at the bottom of the tube. The tubes are held at incubation temperature (36 C) for 14 days before they are reported negative. Viable organisms cannot be recovered 24 hr after inoculation nor after the color reaction has moved to completion due to the accumulation of ammonia. Shepard's U9 medium, as reported, is prepared as follows (18):

BASAL BROTH (pH 5.5)

Tryptic Digest Broth Powder	0.75 g
Sodium chloride ACS	0.50 g
Potassium acid phosphate (KH ₂ PO ₄ , ACS, 0.0015M)	0.02 g
Distilled water or deionized water	100.00 ml
Adjust reaction to pH 5.5 with 1N HCl.	
Sterilize in autoclave at 121 C for 15 min.	

COMPLETE MEDIUM (pH 6.0)

Sterile basal broth, pH 5.5	95.0 ml
Sterile unheated normal horse serum	4.0 ml
Sterile 10% urea stock solution (filter sterilized)	0.5 ml
Sterile 2% phenol red stock solution (autoclave sterilized)	0.1 ml
Penicillin G potassium, 100,000 units/ml stock solution	1.0 ml
Final volume	100.6 ml
Store in refrigerator	

Dispense 2.0 ml volumes of complete U9 medium in 13×100 mm screw-cap culture tubes. Inoculate with urethral exudate (scrapings) or centrifuged urine sediment (0.1 ml). Incubate at 36 C with screw-caps tightened. Include a control tube.

Differential Agar Medium A-6 for Primary Isolation of T-strain Mycoplasmas

A significant advance in T-strain research was the development by Shepard and Lunceford of a differential agar medium labeled A-6 for the direct isolation and immediate identification of T-strain mycoplasmas from clinical material (19). The incorporation of manganous sulfate and urea in this medium provides reactants that selectively color the T-strains. Other classical species which may grow on this medium produce colonies of normal appearance and color, whereas T-strain colonies are a dark, golden-brown. When a sectioned agar block is covered with Dienes stain the golden-brown color

MEDIA

of the T-strains remains unchanged whereas other mycoplasmas stain blue.

The A-6 medium of Shepard and Lunceford was designed for *primary* isolation of T-strains from clinical material such as urethral exudates, urine, etc. Cultures which have been stored frozen for long periods may not produce the typical color reaction; however, broth passage, with accompanying active growth can be expected to restore urease activity and typical coloring.

BASAL MEDIUM (pH 5.5)	
Distilled/deionized water	163.0 ml
Trypticase Soy Broth Powder	4.8 g
Manganous sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$) ACS	0.06 g
Dissolve and adjust reaction to pH 5.5 with 2N HCl	
Ionagar No. 2	1.74 g

Place the container directly in the autoclave and sterilize at 121 C for 15 min. Cool at 56 C in a water bath. Mix contents by rotating container on a flat surface 10–15 sec. This action disperses the melted agar on the bottom. Convert to Complete Medium as follows:

COMPLETE MEDIUM (pH 6.0)	
Sterile basal medium	160.0 ml
Sterile unheated normal horse serum	40.0 ml
Fresh yeast extract, sterile	2.0 ml
Sterile 10% urea stock solution (filter sterilized)	2.0 ml
Penicillin G potassium, 100,000 units/ml	2.0 ml

Mix contents thoroughly by rotating the vessel for 10 sec. This volume of 206 ml is enough for 10 plates, 20 ml/plate, of standard 100×15 mm dimensions. Allow plates to remain on bench inverted overnight before storing in the refrigerator.

COMMENTS ON A-6 MEDIUM

The adjustment of the basal medium to pH 5.5 will result in complete medium of pH 6.0 if enriched with horse serum of about pH 7.3. If the horse serum reaction is higher or lower than 7.3 the basal medium will have to be readjusted above or below pH 5.5 to obtain the desired pH 6.0 final reaction. Thallium acetate is not to be included since it blocks urease activity and growth of T-strains in primary cultures on A-6 medium.

Other T-Strain Media

Alternate media for the isolation and growth of T-strain mycoplasma have been described. The A-2 formula of Shepard may be used, but, like others,

does not differentiate T-strains from other mycoplasmas. Others may be found (20, 21).

Transport Media

While several transport or holding media are described in the literature, little information has been offered on the viability of mycoplasmas during the interval that a clinical specimen is transported to the laboratory. Kenny suspends throat swabs in 2–5 ml of Trypticase Soy Broth containing 0.5% bovine serum albumin (22,23). A 0.1 ml volume of this material is then inoculated into a tube of diphasic medium. Shepard has used 0.25 ml of Tryptic Digest Broth (TDB), pH 7.6, as a carrying medium for the genital T-strains (24). Allen, *et al* used a transport medium consisting of Bacto-agar, 0.75%; Bacto-sodium thioglycollate, 0.075%; phosphate buffer (pH 7.2–7.4), 0.01 M; and sodium chloride, 0.15 M (25). Hendley and Jordan described a carrying medium consisting of PPLO broth supplemented with 10% horse serum, 10% yeast extract, 1% urea, and penicillin (26).

Using cotton swabs and Stuart's Transport Medium (BBL), Ellner and Ellner investigated survival of various bacterial cultures (27). In connection with this work, they also obtained information on a laboratory strain of *M. hominis* (28). When streaked at 0 hour this strain showed 140 colonies; at 2 hours, 124 colonies; at 4 hours, 106 colonies; at 6 hours, 91 colonies; and at 48 hours, only 12 colonies.

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ISOLATION

Isolating Mycoplasmas From Clinical Material

Virtually all types of clinical specimens can be cultivated for the primary isolation of mycoplasmas. Material from the oropharynx and nasal cavity such as cotton swabings, sputum, bronchial and nasal secretions or washing, tooth scrapings, material from dental root canals and the gingival sulcus can be inoculated by streaking or smearing directly onto agar medium. Other materials that have been subjected to examination for mycoplasmas include peripheral blood, bone marrow, urethral secretions and scrapings, anal swabs, tissue fragments, urinary sediment, pus, feces, and exudates from the eye.

The isolation of *M. pneumoniae* is usually carried out by streaking throat swab material directly on the agar surface. The plates are sealed with paraffin to prevent dehydration and then incubated at 37 C in normal atmosphere. They should be examined at frequent intervals, *ad lib*, and not called negative until 30 days of incubation. Other species do best in an atmosphere of 95% nitrogen and 5% carbon dioxide in sealed jars, using unsealed plates. Incubation temperature for species other than *M. pneumoniae* is 37 C. Plates should be examined on the 7th and 14th day, after which they are called negative. An alternate method, that of Kenny, employs diphasic medium and a different incubation system (see page 15). Specimens may be placed in fluid medium, incubated 6 days, and then transferred to agar for demonstrating colonies. If body fluids, tissue culture fluid, or broth cultures are inoculated onto agar medium a 0.1 ml volume is used for 60 mm plates and a 0.3 ml volume is used for 100 mm plates. The inoculum is spread with a bent glass rod or streaked with a loop. It may be spread by tipping the plate to allow the deposited fluid to flow over the surface of the agar.

Isolating Mycoplasmas from Tissue Culture

The problem of mycoplasma contamination of tissue cultures has been known since 1956 when Robinson and colleagues reported that HeLa cells and a line of human conjunctival cells were contaminated with mycoplasmas even though grown routinely in the presence of penicillin and streptomycin (1). Every laboratory utilizing tissue cell lines suffers this contamination, and the best evidence of mycoplasmas in these cultures is to demonstrate the colonies by direct isolation.

PROCEDURE

Step 1. Inoculate 2 agar plates (60×15 mm) with 0.1 ml of tissue culture fluid and cells. Streak with a loop. Seal 1 plate with paraffin and incubate at 37 C in normal atmosphere. Without sealing the 2nd plate incubate in an atmosphere of 95% N₂-5% CO₂. Use standard *M. pneumoniae* medium (page 14) for both plates. Examine at 7 days, 14 days, and 30 days.

Step 2. Inoculate a broth tube containing 2 ml complete PPLO fluid medium with 0.1 ml of specimen. Incubate at 37 C for 6 days. Transfer 0.1 ml broth to each of 2 plates and streak with a loop. Incubate 1 plate aerobically and the other under 95% N₂-5% CO₂ gases as above.

Two-Plate System for Isolating Oropharyngeal Mycoplasma Flora

Because atmospheric, nutritional, and pH requirements vary with species, the use of a single plate cannot be expected to detect all of the strains occurring in man. In response to this problem a 2-plate system has been utilized for routine studies of the oropharynx (2, 3). The first plate, containing medium outlined on page 15, is streaked using only 1 side of the swab and is intended for species other than *M. pneumoniae*. The second plate, containing selective medium for *M. pneumoniae* (page 14), is then streaked with the untouched side of the swab. This technique is outlined in the table below:

AGAR PLATE I		AGAR PLATE II	
SPECIES SUPPORTED			
M. salivarium, M. orale		M. pneumoniae	
M. hominis I, M. fermentans			
INGREDIENTS IN EACH LITER			
see page 15 for preparation		see page 14 for preparation	
PPLO agar	34.0 g	PPLO Agar	23.80 g
Thallium acetate	0.5 g	Thallium acetate	0.35 g
Yeast autolysate powder	10.0 g	Yeast extract aqueous 25%	100.0 ml
Horse serum	100.0 ml	Methylene-blue chloride	0.2 g
Water	900.0 ml	Water	700.0 ml
Penicillin	1,000,000 units	Horse serum	200.0 ml
		Penicillin	2,000,000 units
Amphotericin B	5.0 mg	Amphotericin B	5.0 mg
Final pH 6.0-7.0		Final pH 7.8-8.0	

INOCULATION

One side of the throat swab is rubbed directly on the agar surface of Plate I at the patient's bedside. The cotton tip is inverted before streaking the 2nd plate. Fluid specimens are transferred onto the agar surface with either a pipette,

an inoculating loop, or by saturating a cotton swab which is then rubbed on the agars. Viscous material may be liquified with pancreatic dornase, 0.2%.

AGAR PLATE I

AGAR PLATE II

INCUBATION

Anaerobic; in 95% nitrogen—5% carbon dioxide atmosphere at 36—37 C for 14 days. Examine on 7th day and 14th day.

Aerobic; 36—37 C for 30 days in sealed plates; examine *ad lib* or on 5, 10, 15, 20, 25, 30 days. Aerobic incubation important

COMMENTS

Fungal contamination in anaerobic jars is less frequently observed on this medium.

On primary isolation, under aerobic conditions, this medium is selective for *M. pneumoniae* when prepared with 0.002% methylene blue. Without methylene blue, all species are supported.

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ATMOSPHERES FOR INCUBATION

Establishing 95% Nitrogen—5% Carbon Dioxide Atmosphere

As described in an earlier section, classical mycoplasma species, other than *M. pneumoniae*, are isolated under anaerobic or microaerophilic conditions in the presence of carbon dioxide. Barile and colleagues have called attention to the value of an atmosphere of 95% N₂ and 5% CO₂ gas (1). In such an environment, the number of isolations from clinical material is sharply increased over the number obtained by incubating agar plates in the normal atmosphere. T-strain mycoplasmas do not grow well in 5% CO₂ but do well in atmospheres containing 10–20% CO₂ in either nitrogen or air. The figure presented below illustrates an apparatus for establishing a N₂–CO₂ atmosphere.

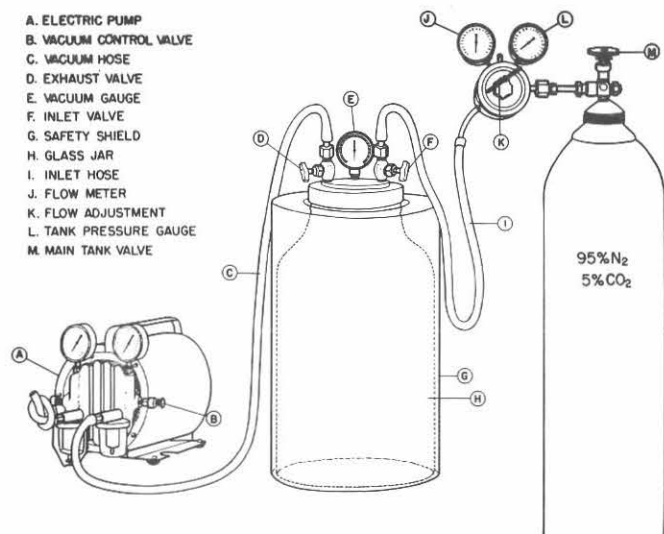


Fig. 11. Device for preparing and incubating mycoplasma cultures in an atmosphere of 95% nitrogen - 5% carbon dioxide gases.

PROCEDURE

The unsealed agar plates are placed inverted in the "Case" jar in stacks of 4–6 plates. Stacks are held together loosely with rubber bands. With the lid firmly tightened, the metal safety shield "G" is placed over the jar as a protective measure against accidental explosion, implosion and flying glass. The hoses, "C" and "I" are then connected. At this point, all valves should be turned to "off" position before the following steps are taken for the completion of one cycle:

1. Open exhaust valve "D."
2. Open main tank valve "M." Leave in open position for 3 cycles.

3. Open vacuum control valve "B" counterclockwise.
4. Turn on vacuum pump "A."
5. Slowly tighten vacuum control valve "B" until needle on vacuum gauge "E" begins to move. The desired vacuum is 20 in. Hg.
6. Close off valve "D" and open "B" to break vacuum, thus reducing strain on motor.
7. Open inlet valve "F."
8. Tighten flow adjustment valve "K" by turning right until needle on flow meter "J" reads 8 liters/minute.
9. When vacuum gauge "E" moves to zero position, loosen flow adjustment valve by turning to left and simultaneously close inlet valve "F." Positive pressure is to be avoided.

The above cycle is customarily performed 3 times. Then all valves are closed, hoses are disconnected and the jar is placed in the incubator.

COMMENTS ON THE ABOVE SYSTEM

1. Fungal contaminants are to be expected in PPLO agar plates when incubated as described above. Decontamination steps are necessary each time a jar is opened.
2. Moisture is well retained by the medium when incubated in the sealed jars.
3. The glass jars will not withstand the autoclave.
4. Hose connections may break if pressure from flow adjustment is excessive.
5. It is a good practice to keep an extra tank of gas in reserve.
6. Vacuum and gas hoses may be fitted with filters to hold down contamination.
7. If vacuum gauge "E" is removed, the screw cap can be sterilized in the autoclave. Vacuum gauge "E" may be unscrewed and connected with a hose and filter to the lid during operation.

Fortner Incubation Method for T-strain Mycoplasmas (Shepard Modification)

In 1928 Fortner described a method for obtaining reduced oxygen, elevated carbon dioxide, and rich moisture content in sealed plates containing *Serratia marcescens* (2). This system has been used over the years by certain workers for growing myco-

plasmas as well as L-forms of bacteria. The Fortner method has been used by Shepard for the isolation and subcultivation of T-strain mycoplasmas. The advantage of this method over the glass jar nitrogen-carbon dioxide system is that the sealed plates may be removed singly from the incubator for examination without breaking the seal. The idea is to grow the aerobe, *S. marcescens* on a separate piece of agar in the same plate as the specimen. The specimen and the aerobe can be manipulated in various ways. The following technic has been kindly submitted by Dr. Maurice Shepard for the isolation and growth of T-strain mycoplasmas.

Step 1. The medium used for propagating *S. marcescens* is Heart Infusion Agar (Difco). Prepare the medium as directed by the label on the container except add 1% dextrose. Dispense and sterilize in 200 ml volumes which is enough to pour 10 plates of 100×15 mm size.

Step 2. Inoculate a plate of medium from step 1 with *S. marcescens* by loop streaking to establish a heavy growth over the entire surface. Incubate at room temperature for 24–48 hr. Maintain culture by making weekly transfers.

Step 3. Cut plate of Dextrose Heart Infusion Agar medium into eight equal sections with a sterile blade. Remove the lid from the plate of agar medium inoculated with the specimen. Place the bottom of this plate in the inverted position on the work bench. Place the top, or cover, in the upward position beside the bottom half.

Step 4. Transfer a section of the Heart Infusion Agar from Step 3 to the center of the exposed lid of

the specimen plate using a flamed scalpel blade. With the same blade inoculate this agar section very heavily with *S. marcescens* from Step 2. Place the bottom of the plate over the exposed top.

Step 5. With the mycoplasma plate still in the inverted position fit an 11-inch length of wrapping twine between the side of cover and bottom of the plate and push it down. Seal with molten paraffin. The twine is pulled to break the seal when opening the plate. Incubate at 36 C for 48 hr in the inverted position.

Note: An alternate method for T-strain incubation used by Shepard is an atmosphere of 15–20% carbon dioxide in nitrogen or air.

Comment on T-strain incubation: An atmosphere rich in carbon dioxide is critical for isolation and growth of T-strains. Ford concluded that an atmosphere containing 5% carbon dioxide or candle jar incubation was inadequate, and that atmosphere of 10% carbon dioxide was superior to Fortner's method (3).

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MICROSCOPIC STUDY OF MYCOPLASMA

Low Magnification

The details of colony structure are best revealed by making a direct microscopic examination of the agar surface. The sealed plate is inverted on the stage and colonies are brought into view under low power magnification (60–100X). Under these conditions, the growth is seen in its natural position on the surface and in the agar. The preliminary examination of agar plates as in screening operations is usually performed with a dissecting microscope using 10–20X magnification and oblique illumination.

High Magnification

The study of agar cultures under higher magnification is performed with agar blocks mounted in the upright position on a glass slide as reported by Dienes and also by Madoff (1, 2). A stained or unstained coverslip is placed over the agar section and the edges sealed with a mixture of 1 part vaseline and 9 parts paraffin to prevent dehydration. The preparation of an agar mount of this type is illustrated in Figure 12. If stained coverslips are used, the mature, well-isolated colonies usually be-

come bright blue, whereas densely growing colonies tend to stain less brightly.

Agar Impressions

Another technique utilizes agar impression slides. These are prepared by placing a block of agar, colony-side down, on a defatted polished slide; after 2–3 min the slide is held upside down as the block is flicked away with a fine-pointed instrument. After fixing the slide for 10 min in absolute methanol, the colonies clinging to the slide can be colored with Giemsa stain.

Hot Water Fixation

A hot water fixation method of preparing mycoplasma colonies for microscopic examination was described by Clark, Fowler, and Brown (3).

1. Locate colonies under microscope and mark 1 cm² area on plate.
2. Cut out section and place on glass slide with colonies against glass.

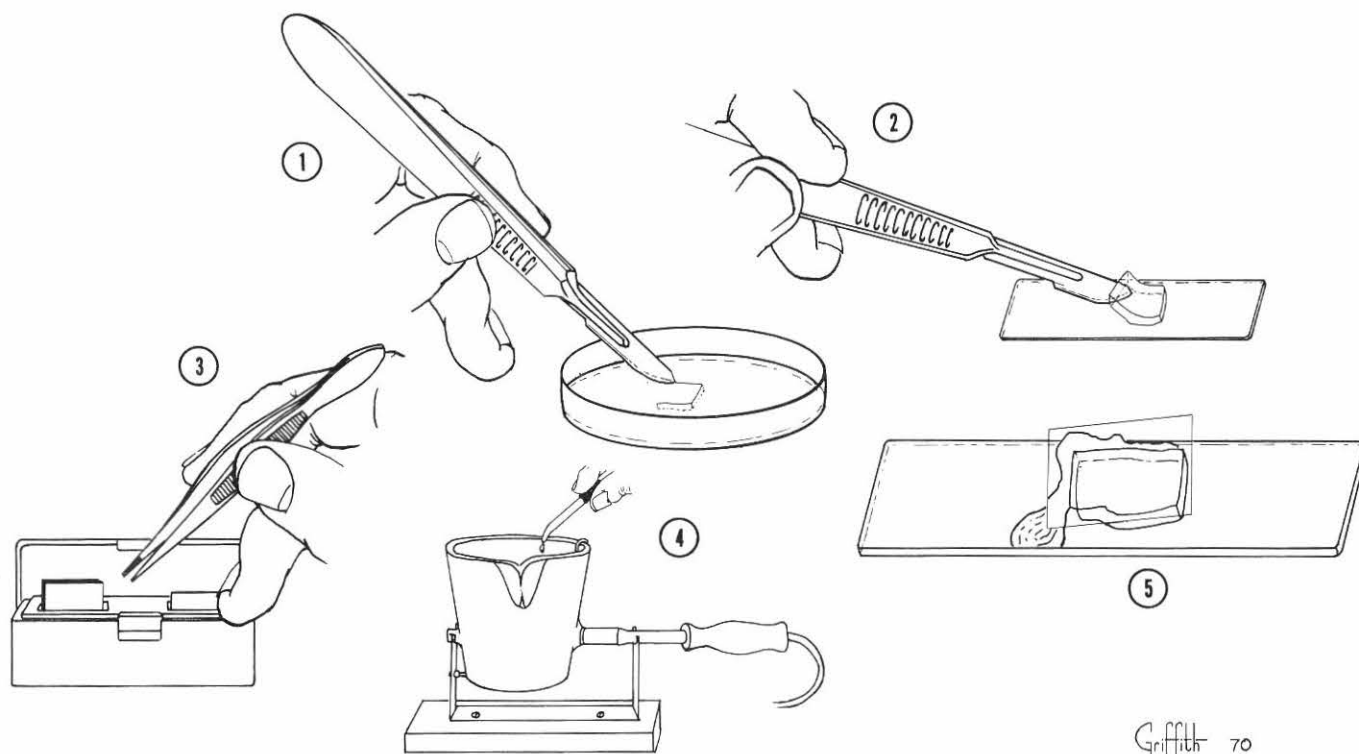


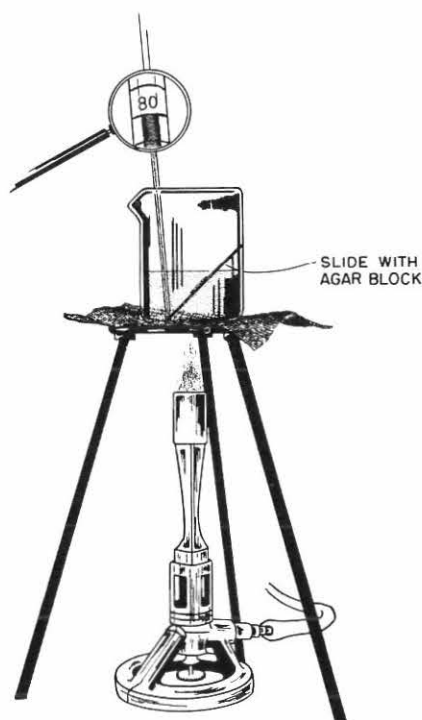
Fig. 12. Preparing an agar mount for the microscopic examination of mycoplasma colonies; (1) cutting an agar block (2) laying agar block upright on glass slide (3) selecting a coverslip which is placed over the block and sealed with paraffin (4,5).

Griffith 70

3. Immerse slide in a beaker of water at 80 C. Angle of slide should be 45° as it leans against the beaker wall.
4. Raise water temperature rapidly without agitation using Bunsen burner.
5. When agar block slips off slide or melts away, remove slide.
6. Rinse with distilled water and allow to air-dry.

Miscellaneous Techniques

A number of authors have reported success with more complex procedures, such as darkfield illumination of solid and liquid media, fixing and staining of Formvar film, acridine-orange staining, and with annular oblique illumination techniques (4).



Griffith 70

Fig. 13. Hot water fixation of mycoplasma colonies for microscopic examination.

Pseudo-Colonies

A possible source of error in microscopic examination is caused by precipitated crystalline materials that resemble mycoplasma colonies on the surface of serum agar (Fig. 14). These pseudo-colonies develop best on plates enriched with 30% normal horse serum, but plates containing rabbit, human,

and bovine serum also form these interesting structures. Individual peculiarities are imparted to the "colony" by each kind of serum. Appearance of these artefacts which occurs either at 37 C or room temperature is not influenced by the presence of merthiolate. Heating the medium to 70 C for as long as 1 hr does not prevent subsequent development. If one or two "colonies" are transferred by agar block to a fresh plate, very many new ones aggregate in the inoculated area, leading to the belief that a microbe has been isolated.

The pseudo-colony, according to early authors, is formed by precipitation of spherocrystals around a so-called "Amber Body" (5, 6, 7). Chemical tests and differential staining techniques suggest that these artefacts are composed of a fatty substance that precipitates from the medium as calcium and magnesium soaps.

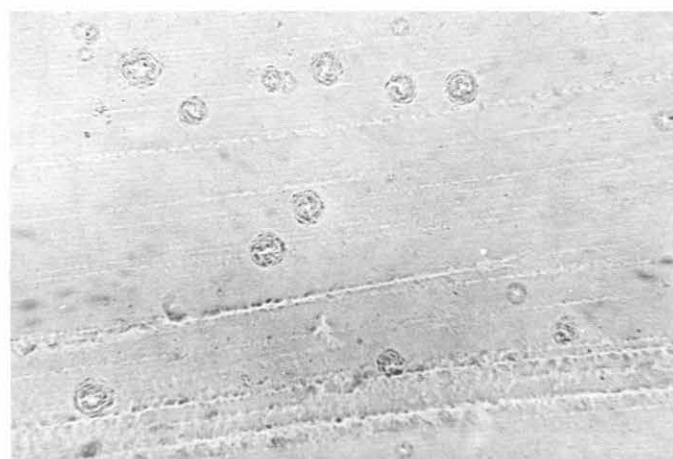


Fig. 14. Pseudo colonies on surface of PPLO agar medium. The agar had been streaked with a cotton tipped swab bearing material from the pharynx of a germ-free mouse. (X30).

Pin-Wheels

Another colony-like structure regularly develops on the surface of PPLO agar plates inoculated with pharyngeal material. The appearance is that of a "pin-wheel," an artefact produced by epithelial cells (Fig. 15). Bonifas and associates interpreted these structures as microorganisms and labeled them "Stegasma" (8, 9). Organick studied them in detail, traced their development during incubation, and called them artefacts (10). Epithelial cells resting on the surface of serum-enriched PPLO agar develop 1, 2 or more round black dots. These dots increase in numbers and size by the 10th day, and somewhat later, a change from blackness to a yellowish green color will be noted. Between the 14th and 28th day curled, hair-like projections appear, which impart

the "pin-wheel" appearance to the artefact. These bodies develop in all pharyngeal cultures, whether derived from specimens from healthy or diseased individuals, and 80% to 100% of the epithelial cells spread out on the agar will produce them.

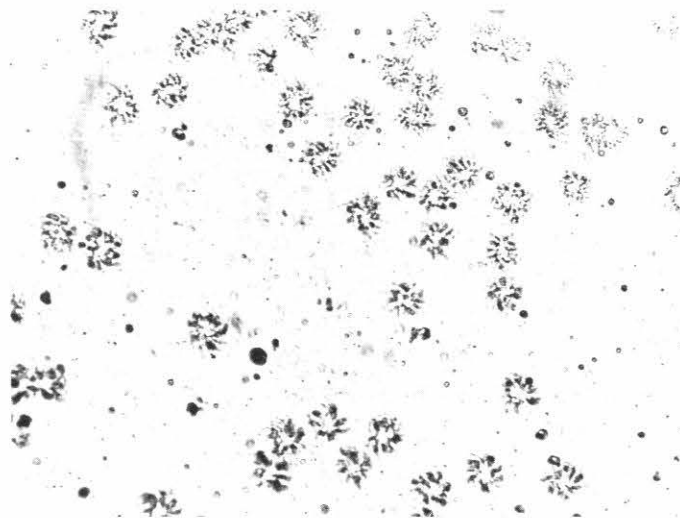


Fig. 15. "Pin-wheel" colony-like structures on the surface of PPLO agar medium. (X30).

Artefacts in Broth

Other artefacts occur in broth cultures, especially of blood, and these, too, have tripped the unwary. Tubes of broth, although sterile, often develop coccoid bodies, spherical elements and fibers. When stained or suspended in hanging drops, such materials are indistinguishable from the L-forms of bacteria (11). That a sediment forms in sterile broth tubes when incubated for several days can be demonstrated by flicking the tube and observing the swirl which arises from the bottom. Nelson's experiments with blood cultures indicate that these artefacts are formed by erythrocytic lysates and hemoglobin which organize into particles in the presence of certain compounds in peptone (12). Artefacts of this type appear randomly in cultures of blood, and have been reported by many authors as microbic

growth. Broth-to-broth transfers, resulting in renewed turbidity, may lead one to believe that a microorganism is present; however, if the turbid material is transplanted to solid medium no growth occurs.

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SUBCULTIVATION

It has been shown that 2 or even 3 species of mycoplasmas often grow out in primary cultures of the oral region (1). It is a disturbing fact also that a single colony on a primary plate can consist of 2 species (1, 2). Hence, it is not safe to assume that a group of colonies, or 1 colony in a primary culture, is pure. Single colony cloning procedures will separate these mixtures and any one of several methods may be used.

Agar to Agar by Needle and Loop

1. Uncover dish and place upright under dissecting microscope.
2. Select well-isolated colony using 20-40X magnification.
3. With a rigid 1.5 cm platinum wire, previously sterilized and cooled, puncture and stir the colony (Fig. 16).

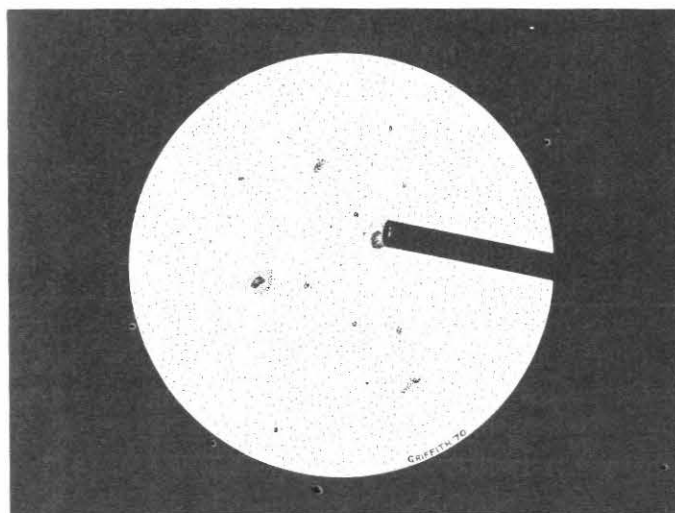


Fig. 16. Puncturing a mycoplasma colony with a needle under a dissecting microscope. (X20).

4. Transfer to fresh agar medium by streaking 3 or 4 short parallel lines from edge to center of plate (Fig. 17a).
5. Substituting a loop for the needle, streak the parallel lines at right angles, streaking across the entire plate (Fig. 17b).
6. To insure purification, this process must be carried out twice and for a margin of safety, 3 subcultivations should be made.

Comments: A short rigid needle (1.5 cm) is preferred over longer needles which are wobbly and difficult to control under the microscope. Agar cultures of classical species such as *M. salivarium* and *M. orale* remain viable at room temperature and

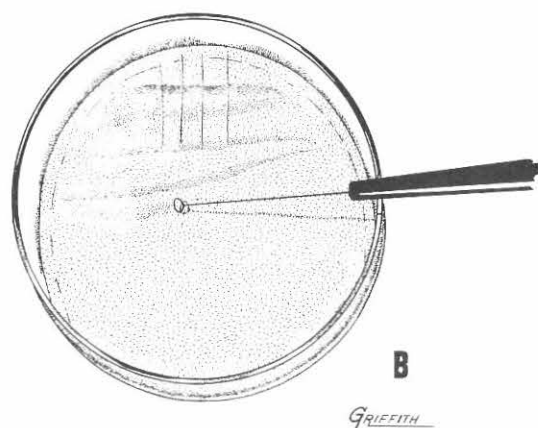
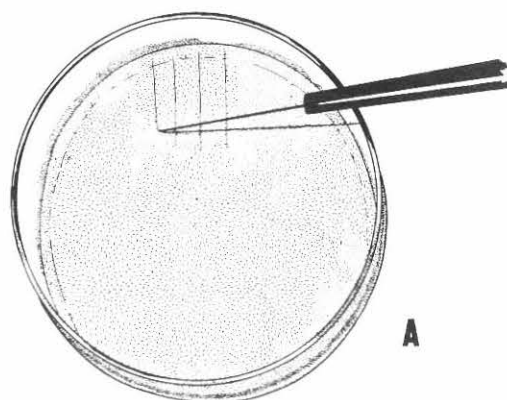


Fig. 17. Streaking out a single colony of mycoplasma. A series of parallel lines are made at A. These lines are then cross streaked with a loop at B.

may be subcultivated after 3 or 4 weeks on the bench. Single freshly isolated colonies may be transferred and streaked using only a loop; however, older cultures often fail to grow when transferred by this method. Other workers have attempted purification by cutting out an agar block with but one colony on the surface; however, it can be argued that the sectioned agar surface may harbor unseen, non-proliferating organisms still capable of multiplication if stimulated by transfer.

Agar to Agar by Block Transfer

Agar cultures may be transferred to fresh plates by cutting out a piece of agar with colonies on the surface using a flamed blade, small spatula, or spud. The inverted block is placed on fresh agar medium, and pushed over the surface (Fig. 18). Pressing the section of agar downward to insure firm contact is

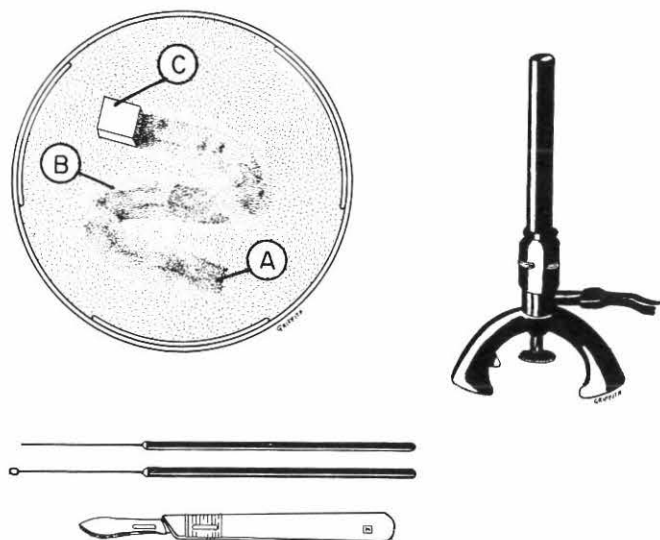


Fig. 18. Seeding a plate by agar block. The block is placed face down at A, pushed over area B, and allowed to rest at C. If growth fails, the area below point C should be passed along with the original block.

an important measure, especially if a newly isolated strain is being passed. The inoculating block should be allowed to rest on the agar surface throughout incubation as growth may occur only at its edge or interface.

If the first passage does not produce new colonies, the original block can be passed to a second plate. It may be rewarding to remove a section from where the block has rested, and pass it inverted onto the new plate.

Agar to Broth by Pasteur Pipette

An alternate method of transferring single colonies was described by Morton and Roberts (3). A well-isolated colony is selected with the unaided eye. A small volume of sterile saline is taken up with a Pasteur pipette fitted with a tube and mouthpiece. The tip of the pipette is then used to "punch-out" the colony while applying suction to draw the colony into the pipette. The colony is then blown into a tube of fluid medium (Fig. 19). Following incubation of the inoculated broth the organisms can be plated for a second purification by repeating the procedure.



Fig. 19. Pasteur pipette method of transferring colonies from agar to broth.

Agar to Broth by Agar Blocks

The inoculation of fluid medium from an agar culture is easily performed. An agar section with densely growing colonies is removed from the plate and dropped into a broth tube or flask using aseptic precautions. While one block is usually sufficient for tube cultures, more than one block should be used for initiating growth in flasks containing larger volumes of medium. These agar sections sink to the bottom and may be used as an indicator of growth, i.e., colonies on the block enlarge as multiplication in broth proceeds. Fluid cultures often remain clear to the eye even though as many as 10^5 organisms per ml are present. Well-colonized agar sections can be prepared for seeding broth by using the method illustrated in Fig. 20. A loop from a single colony is transferred to fresh agar and rubbed vigorously in a circular area the size of a dime. Several areas can be smeared in the same plate if a pure culture is being processed. After 5 to 7 days, or when abundant growth appears, cutting out these agar plugs insures a heavy inoculation when dropped into fluid medium.

Mycoplasma pneumoniae cultures are transferred on the 7th day of incubation while other species are customarily transferred after 72 to 84 hr.

Broth to Agar by Pipette or Loop

Fluid cultures may be transferred to solid media by transferring 0.10 ml to 60×15 mm plates, and 0.30 ml to 100×15 mm plates. The surface of the agar may be evenly seeded by smearing the surface with a sterile, bent glass rod. An alternate method of spreading the organisms is to tilt the plate several times allowing the liquid to flow downward by gravity until the entire surface is covered.

If well-isolated colonies are desired, the transfer from fluid to solid medium may be made with a loop. A thorough streaking across the entire plate of 1 loopful of log phase material yields a good supply of scattered colonies.

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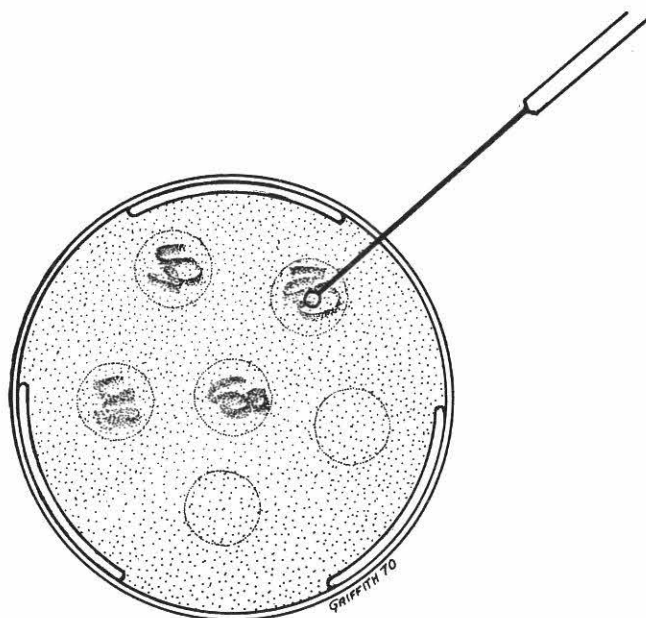


Fig. 20. Preparing agar blocks with heavy inocula for seeding broth tubes.

Broth to Broth by Pipette

When propagating mycoplasmas in fluid medium the transfer is customarily made with a sterile pipette. The volume of inoculum ordinarily used is 5.0 ml of log phase culture per liter of broth.

IDENTIFICATION AND CHARACTERISTICS OF HUMAN STRAIN MYCOPLASMAS

A number of biophysical properties, and biochemical and serological reactions of mycoplasmas can be used for their species identification (1). While the clinical laboratory worker may wish to concentrate his efforts on the characteristics of *Mycoplasma pneumoniae*, a fundamental knowledge of other species inhabiting man is equally essential. Of the biochemical reactions produced, it is useful to remember that dextrose degradation and arginine breakdown classify the human strain mycoplasmas into 3 groups: (a) dextrose positive-arginine negative (*M. pneumoniae*); (b) dextrose positive-arginine positive (*M. fermentans*); and (c) dextrose negative-arginine positive (all others). The first step in attempting identification of an unknown species is the microscopic examination which hopefully might provide a presumptive identification of *M. pneumoniae*.

Microscopic Recognition of *M. Pneumoniae*

A presumptive identification of *M. pneumoniae* can be made microscopically on standard medium even when colonies are as small as $0.5\ \mu$. The typical colony is refractile, spherical in shape with a finely granular surface, and golden hue. Fully mature cultures reveal colonies ranging in size from $10\ \mu$ to $250\ \mu$. The most consistent feature on the standard agar medium is absence of typical fried-egg form because the organisms do not spread outward on the agar surface from the central core (Fig. 21). Exceptions to this picture show the outer fringe as a

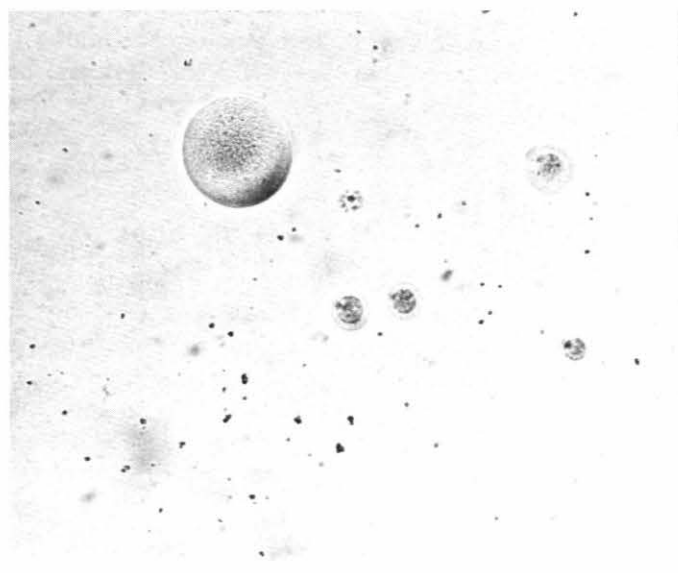


Fig. 21. Colonies of *M. pneumoniae* strain GL-M52 on agar surface. The large colony lacks the surface fringe which is a characteristic of growth on standard medium. (X100).

very narrow band. The most likely source of error is to confuse *M. orale* type 1 with *M. pneumoniae* since the former also may lack the outer peripheral zone in primary cultures. Clyde and Kim have reported on the biophysical properties of mycoplasmas (2). In their hands, *M. orale* type 1 could be distinguished from *M. pneumoniae* by the darker brown coloration of the *M. orale* core, and the irregular contour of the fringed area. Photographs of other species further illustrate the points stressed above (Figs. 1-6).

Beta-hemolytic Plaque Test for Identification of *M. pneumoniae*

The hemolytic plaque test recommended by Clyde, and also by Somerson, Taylor-Robinson and Chanock has been used to differentiate *M. pneumoniae* from other mycoplasma species of human origin (3, 4). According to the experience of these authors, and under the conditions of the test, *M. pneumoniae* colonies produce clear zones of beta-hemolysis. In contrast, other species of human origin produce alpha- or gamma-hemolysis. A few exceptional strains of *M. fermentans* however, have been observed to produce beta-hemolysis (2). Strict attention must be given to the exact procedure if valid results are to be obtained.

STEP 1. GROWING THE CULTURE

An agar culture of the mycoplasma in question is grown on the standard *M. pneumoniae* agar medium (page 14). Agar block inoculation of the test plate should be avoided because very crowded colonies give falsely negative hemolysis. Plates streaked with a loop or prepared by inoculating and spreading 0.1 ml of liquid culture dilutions, i.e., 10^{-1} , 10^{-2} , 10^{-3} , will result in well-spaced colonies that hemolyze more actively. The plates are then incubated in the usual manner until colonies first appear (5 to 6 days).

STEP 2. PREPARING THE OVERLAY

The blood agar overlay can be poured when the colonies first become apparent, or as late as 4 to 5 days afterward. Either guinea pig or sheep blood in Alsever's solution may be used; however, sheep erythrocytes are preferred since they do not lyse as rapidly in the medium as those of the guinea pig which often lyse from temperature change, i.e., placing the plate on the bench after overnight incubation at 37 C .

One percent Bacto-agar is prepared in Alsever's solution and sterilized by autoclaving. When cooled at 45 C, 3 parts of this solution are added to 1 part of 50% blood-Alsever's mixture. After the blood is mixed by rotating the flask, or tube, a thin layer is poured over the colonies. Tipping the plate slightly allows an excess to accumulate at one edge leaving a very thin layer covering most of the colonies. After the agar has solidified, the plates are sealed with paraffin and placed in the 37 C incubator for daily observation. Incubation should be continued at 36-37 C for a minimum of 4 days before the test is recorded as being negative.

STEP 3. READING THE TEST

Hemolysis may occur in 18 hr and zones should be examined microscopically under low magnification. The zones surrounding the colonies are usually visible to the unaided eye, but details of cell lysis are best viewed with the help of a microscope. *Mycoplasma pneumoniae* colonies are surrounded by clear zones of sparkling hemolysis, while other species from man, with exceptions mentioned, are reported to produce no hemolysis, partial hemolysis, or alpha-hemolysis.

ERRORS OF THE HEMOLYTIC PLAQUE TEST

Type of Error	Cause
1. Hemolysis fails to occur (false negative).	Colonies crowded; medium lacks yeast, aerobiosis.
2. Lysis occurs throughout medium before zones are apparent.	Autolysis, hot-cold lysis. Guinea pig RBC most susceptible.
3. False positives.	Possible <i>M. fermentans</i> .

Investigations of the hemolysin elaborated by *M. pneumoniae* have shown that it is labile, low in molecular weight, and that it continuously exudes from the colonies (5). If a dialysing membrane is placed above the colonies and below the blood agar overlay, hemolysis still proceeds. A nutritionally rich medium has been found essential for obtaining the complete reaction; for example, if the medium contains no yeast extract, alpha-hemolysis results. Anaerobic incubation is inhibitory, while aerobic conditions reverse this inhibition. *Mycoplasma laidlawii* also produces beta-hemolysis under the same conditions. Further studies by Somerson and associates have indicated that hemolysin of *M. pneumoniae* is a peroxide (6).

Note: For added information and another technique, see Cole, *et al* (7).

Hemadsorption Test for Identification of *M. pneumoniae* Colonies

Del Guidice and Pavia observed that *M. pneumoniae* colonies possess hemadsorptive properties

whereas other species of mycoplasmas from human sources do not react (8). The test may be performed within 5 days after colonies first appear on agar. Although the authors noted that erythrocytes of several animal species were sensitive, guinea pig red blood cells were used.

PROCEDURE FOR HEMADSORPTION

1. Flood the colonies with 0.5% erythrocytes in 0.5% Dienes stain.
2. Incubate plates at 37 C for 15 min.
3. Remove the RBC suspension and wash the agar surface with saline.
4. Examine colonies microscopically.

The test can be performed directly on primary isolation plates, and the reaction can be inhibited by treating the colonies with antisera.

PROCEDURE FOR HEMADSORPTION-INHIBITION

1. Inoculate agar plates with 0.2 ml of a 3-5 day broth culture.
2. Incubate at 37 C and use within 5 days after colonies first appear.
3. Prepare serum dilutions in veronal buffer enriched with 1:20 normal guinea pig serum.
4. 1 ml of each serum dilution is added to each plate and then incubated at 37 C for 1 hr. Remove the serum.
5. 1 ml of 0.5% guinea pig RBC in 0.5% Dienes stain is added to plate.
6. Incubate 10 min at room temperature.
7. Remove cells and wash with saline.
8. Examine colonies under the microscope.

Tetrazolium Color Test for Identification of *M. pneumoniae*

The differentiation of *M. pneumoniae* from other mycoplasmas isolated from human beings can be achieved with the tetrazolium color test (9). This reaction has been recommended as a biochemical test for identification of *M. pneumoniae* and is useful if antisera are not available. While *M. pneumoniae* is an active reducer of tetrazolium chloride under aerobic conditions, other species occurring in man do not produce this change unless incubation is anaerobic. The reaction produces a pink color, and is most dramatic when growth on the surface of an inverted agar block is incubated in contact with an agar surface containing the tetrazolium. The color change is specific only if aerobic conditions are used. The specificity and simplicity of the test can be deduced from Fig. 22.

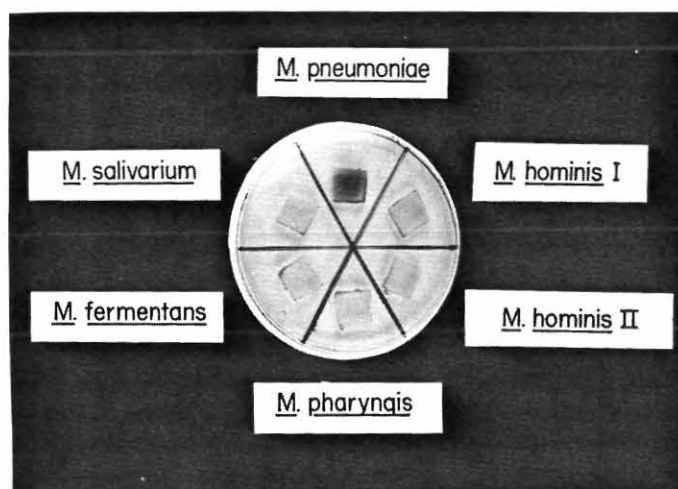


Fig. 22. Tetrazolium color test showing specificity of reaction.

PROCEDURE

STEP 1. STIMULATING GROWTH ON AGAR

It is important that a dense population of colonies is growing before the test is attempted. Freshly isolated strains that may grow slowly often fail to react positively. One or 2 rapid passages on agar will usually stimulate growth. The agar surface should show, as a rough index, 300 or more colonies per cm² before a section is removed for testing.

STEP 2. PREPARATION OF PPLO AGAR WITH TETRAZOLIUM ADDITIVE

A 0.02% concentration of tetrazolium chloride is added to the standard *M. pneumoniae* agar from the stock solution as follows: Just before pouring the medium add 2.0 ml of 1.0% stock solution of tetrazolium to 98 ml of the complete molten agar and mix well.

STEP 3. PREPARING AND INTERPRETING THE TEST

Remove a block of agar with colonies from the plate. Place the block, colonies down, on the tetrazolium agar, push one-half inch, and allow the block to rest. Economy of materials can be achieved by testing as many as 10 specimens on a single 100×15 mm plate (see Fig. 22 which contains 6 specimens). The plate is sealed with paraffin and placed in a 36–37 C incubator. If *M. pneumoniae* colonies are present, the resting piece of agar should develop a pink color in 3 to 6 days. The color change may be slight or it may permeate the entire block. False positives have occurred with human strain mycoplasma; however, as with the hemolytic plaque test, *M. laidlawii* here again produces a posi-

tive response. Thus, it is essential to be informed on the origin of the culture before making interpretations. False negative reactions are thought to be caused by: (a) an insufficient number of colonies under the block on the test plate, or (b) failure to multiply. The latter factor can be determined by examining for colonies in the area over which the test block was pushed. If colonies are absent, very probably growth has not occurred. An error in medium preparation, fluctuations in incubation temperature or non-viable inoculum should be suspected.

Dextrose Utilization for Identification of *M. pneumoniae* and *M. fermentans*

Mycoplasma pneumoniae produces acid but no gas from dextrose, maltose, xylose, mannose, dextrin, and starch. *Mycoplasma fermentans* also attacks carbohydrates, i.e., acid but no gas from dextrose, maltose, dextrin, fructose, galactose, glycogen, and starch. Other species of human origin are non-fermentative. Since *M. fermentans* does not normally occur in the respiratory tract, the fermentation of dextrose may be used as an identifying reaction (a) for *M. pneumoniae*, and (b) for separating *M. fermentans* from *M. hominis* isolates obtained from the genitourinary tract, joints, or other sources.

PROCEDURE OF TEST

Inoculate dextrose broth tubes described on page 16 as follows. Cut an agar section with heavy growth of the unknown colonies previously purified. Cut the block small enough to pass through tube opening. For a positive control cut a block from an agar culture of a known *M. pneumoniae* strain and drop into a second broth tube. A control on the medium should consist of a third broth tube incubated with a sterile piece of agar inoculum. The caps are screwed tightly and incubation is held at 36–37 C.

READING THE TEST

Read tests daily or more often for 2 weeks. A color change from red to yellow in the test and positive control tubes, without similar change in the medium control tube, is a positive reaction. For another procedure, see Aluotto, *et al* (1).

Arginine Utilization for Exclusion of *M. pneumoniae*

Mycoplasma pneumoniae which breaks down dextrose oxidatively does not attack arginine. *Mycoplasma fermentans* which attacks dextrose fermentatively also breaks down arginine. Other spe-

cies of human origin also attack arginine with release of ammonia with an upward shift of pH.

PROCEDURE OF TEST

Inoculate arginine broth tubes described on page 16 as follows. Cut an agar section with heavy growth of the unknown colonies previously purified. Cut block small enough to pass through tube opening. For a positive control, cut inoculating block from known arginine positive mycoplasma and inoculate another tube. A negative control tube is inoculated with an *M. pneumoniae* infected agar block, or sterile agar block. The caps are tightened and incubation is held at 36–37 C.

READING THE TEST

Read tests daily or more often for 2 weeks. A color change to redness or darker red in comparison with negative control tube constitutes a positive test. The positive control tube should show a similar change. Another procedure has been described (1).

Miscellaneous Reactions and Phenomena

The mycoplasmas of human origin, and those from other sources, are capable of producing a number of reactions that are of interest to taxonomists and of assistance in the identification of unknown strains. Certain of these reactions and phenomena are described in Bergey's manual, while methods for their demonstration can be found elsewhere in the literature (1).

Growth on rabbit serum agar: Some weight was formerly placed on the ability of mycoplasmas to grow on media containing rabbit serum, since some animal strains developed poorly on such media. *Mycoplasma hominis*, *M. fermentans*, and *M. salivarium* were observed by Freundt to grow equally well on rabbit serum agar. This attribute has little practical value in differentiating human strain mycoplasmas.

Reduction of methylene blue: The ability to reduce methylene blue to the colorless state was formerly thought to have little practical value for differentiating mycoplasma species. However, in the hands of Aluotto, *et al* this test was of value for separating *M. fermentans* and *M. pneumoniae* which reduce the dye in 24 hr anaerobically (1). Other species do not reduce methylene blue in 24 hr either aerobically or anaerobically. Freundt performed the test by adding a few drops of an aqueous solution of methylene blue (0.3%) to 1- to 2- day old cultures. The cultures were grown in semi-fluid medium at 37 C and reduction was observed in the lower 4 to 5 cm of the tube. Non-fermentative species tended to

react more slowly (after 2 days and only 12 hours before control tubes). Fermentative strains reduced the dye more rapidly, i.e., decolorization in 3 to 12 hr. Aluotto and colleagues used a 0.1% (w/v) stock solution of methylene blue chloride sterilized by filtration through a 0.45 μ Millipore filter. A 0.1 ml volume of this stock solution was pipetted into 2.9 ml of a 24-hr broth culture. One tube was stoppered with gauze (aerobic) while a second tube was overlaid with a vaseline-paraffin mixture (anaerobic). After 24 hr the tubes were read for reduction. A complete decolorization of methylene blue was a positive reaction, a green color was considered a weak positive, and a blue color was regarded as negative. The positive and negative controls were mycoplasmas of known reactivity for methylene blue, i.e., *M. bovirhinis*, ATCC No 19884 (+) and *M. arthritidis*, ATCC No 19611 (—).

Reduction of potassium tellurite: The reduction of tellurite by *M. pneumoniae*, *M. fermentans*, and *M. orale* type 2, has been demonstrated and a method described (1). Plates are prepared by adding 20 ml horse serum, 5 ml yeast extract stock solution, and 0.5 ml of stock 1% (w/v) potassium tellurite solution to 74.5 ml heart infusion broth (organisms already adapted, see ref. 1). Duplicate plates were inoculated, each with two inverted blocks of agar, one of which showed densely growing colonies, and the other well isolated colonies. The plates were incubated aerobically and anaerobically. Microscopic examinations were made at frequent intervals over a period of 2 weeks. A positive reaction was indicated by black colonies. Brown or colorless colonies were called negative. Controls were: *M. bovirhinis* ATCC No 19884 (+) and *M. arthritidis* ATCC No 19611 (—).

Test for phosphatase: A single strain of *M. orale* type 2 has been reported to be phosphatase positive (1). The test is performed on plates prepared by adding 20 ml horse serum, 5 ml yeast extract stock solution, and 1 ml of stock 1% (w/v) solution of the sodium salt of phenolphthalein diphosphate to 74 ml of heart infusion agar (organism already adapted, see ref. 1). Triplicate test plates are inoculated using a drop from a broth culture. After 3, 7, and 14 days respectively, the surface flooded with 5N NaOH. A positive test was indicated by the appearance of a red color. The test control organisms for phosphatase positive and phosphatase negative mycoplasmas were *M. arthritidis* ATCC No 19611 (+) and *M. bovirhinis* ATCC No 19884 (—).

Film and Spots (lipolytic reaction): Edward described film and spots production by mycoplasmas (10). He later showed that this reaction was asso-

IDENTIFICATION AND CHARACTERISTICS

ciated with lipid metabolism. Film is an oily layer appearing only on the surface of the growth. Spots are clusters of dark precipitates on and under the surface of the medium (Fig. 23a and 23b). Cole and Pease investigated the lipolytic activity of oral strains and described technics for making this determination (11). *Mycoplasma salivarium* and *M. fermentans* produce film and spots. In the *M. salivarium* group both lipolytic and nonlipolytic strains exist.

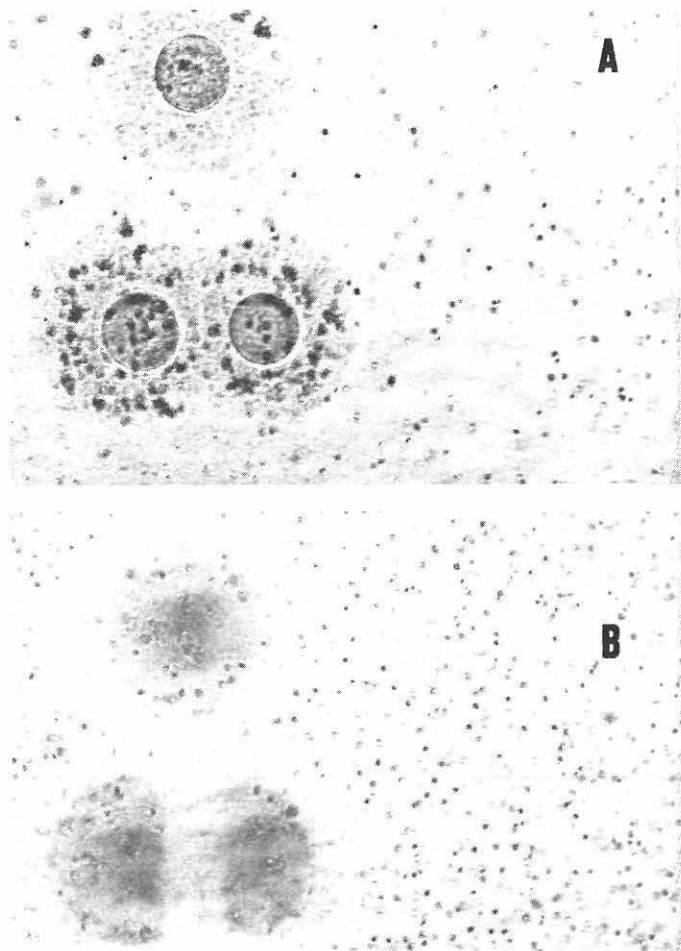


Fig. 23. Spot formation by a *M. salivarium* strain showing (A) spots on the agar surface and (B) subsurface. (X100).

Butter fat and castor oil are added to the solid medium in the form of a 10% (w/v) emulsion in distilled water. The emulsions are prepared by treating the mixture with a Mullard ultrasonic drill. The final concentration in the solid medium is 0.5% (w/v). Tributyrin breakdown is tested on Oxoid tributyrin agar. Tweens 20, 40, 60, and 80 were tested on base agar containing 2% (w/v) Tween and 0.01% (w/v) calcium chloride. Decomposition of Tween was shown by the production of a film over the surface of the growth and precipita-

tion beneath the agar. In another test, egg yolk suspension (Oxoid, SR 47) is added to basal medium using 10% (w/v) concentration. The plates are inoculated and incubated 7 days. Lipolysis is indicated by the development of a film over the growth and precipitation in the agar. Proteolysis is indicated by a wide zone of clearing of the egg yolk emulsion.

Paper-Disc Growth Inhibition Test for Identification of *Mycoplasma* Species

Huijsmans-Evers and Ruys described a technique later adapted by Clyde for the species identification of *Mycoplasma* (12, 13). The observation of Edward and Fitzgerald that inhibition of growth by serum antibody is a species-specific reaction forms the basis of the test (14). Paper discs, impregnated with rabbit antisera, are placed on the surface of PPLO agar seeded previously with the specimen. After 5 to 10 days of incubation, a clear circular zone surrounding a disc in which colonies within the zone are inhibited, or appear greatly thinned, is a positive reaction and identifies the species. The scheme of the test, illustrated in Fig. 24, shows an identifying reaction for *M. salivarium*.

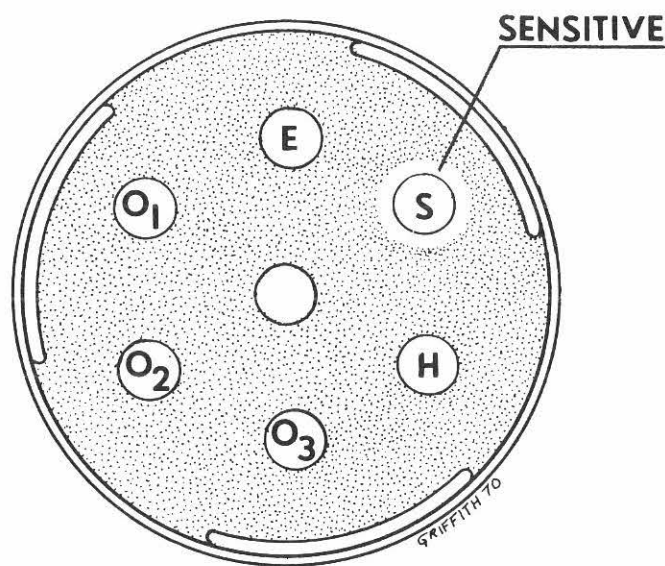


Fig. 24. Paper disc growth inhibition test showing positive *M. salivarium* reaction.

While the procedure is simple and easily performed, the test is subject to errors culminating from moisture, dilution, and diffusion factors. Firstly, to perform this test antisera must be made available, either by immunizing rabbits or by open purchase

on the market (see supply table). One antiserum against each of the common species of human origin must be included in each test to insure the specificity of any reaction obtained.

STEP 1. PREPARATION OF ANTIGEN FOR RABBIT IMMUNIZATION

Twenty ml of mycoplasma antigen is prepared by suspending in saline the sediment from 1 liter of culture. *Mycoplasma pneumoniae* is incubated for 7 days, while other species are incubated 3 to 4 days. An alternate method for *M. pneumoniae* vaccine is to grow the organism on a glass surface (page 16). When the cells have been collected they are washed with sterile physiological saline and then transferred into previously sterilized vaccine bottles. Storage is at 4 C, but storage of 0.5 or 1.0 ml volumes in sealed ampules at -20 C is also convenient. To insure a full component of antigens for rabbit immunization the organisms are not killed.

STEP 2. IMMUNIZING RABBITS

Young, adult New Zealand White rabbits are used. A serum specimen is first tested to insure that growth-inhibiting antibody against each species is absent. *Note*: Inhibition of *M. pneumoniae* by the serum of apparently normal rabbits is not uncommon. The first injection of 0.5 ml is given by ear vein; the second injection is 0.5 ml given intramuscularly; and the third is 0.5 ml administered subcutaneously. This routine of injection is repeated at weekly intervals until the serum shows an inhibiting zone of at least 1.5 to 2.0 mm. Zones of up to 7.0 mm are commonly produced by *M. pharyngis* antisera. Circulating antibody usually appears in the rabbit by the 3rd or 4th week; hence, a small amount of serum may be taken for testing at either time. Immunizations are continued by rotating the site of injection at weekly intervals for as long as the rabbit is maintained. After the 4th week, the dose of vaccine is reduced to 0.25 ml. *Note*: For another technique see Clyde (10).

STEP 3. COLLECTION AND STORAGE OF RABBIT ANTISERA

Once it has been determined that the rabbit has produced antibody of satisfactory potency, one of two courses may be followed: 1) sacrifice the rabbit by collecting as much blood as possible (80-120 ml), or 2) take smaller volumes (40-50 ml) and save the rabbit for further booster injections and bleedings. A greater yield of serum per rabbit will be obtained if option 2 is followed. It is customary to perform a cardiac puncture and allow clotting to

occur at room temperature. After freeing the clot from attachment to the glass, the tube is kept at 4 C overnight. This permits further contraction of the clot and a greater yield of serum will be obtained.

For performing occasional tests with paper discs, a small volume (0.2 ml) of serum in a 1.0 ml glass ampule sealed by flame is convenient and insures safe storage. Screw-cap vials are used for storing larger volumes such as 5 to 10 ml amounts for shorter periods of time. The sealed ampules and screw-cap vials are stored frozen at -20 C. Clyde reported that the growth-inhibiting antibody titer is not reduced even if sera are frozen and thawed through 20 cycles (10).

STEP 4. INOCULATING THE PLATE WITH UNKNOWN

It has been customary to inoculate all test plates from a 48-72 hr broth culture of the unknown. This step is still advisable for cultures presumptively identified as *M. pneumoniae*. For species other than *M. pneumoniae* the modification in the procedure outlined below saves 2-3 days of incubation.

Step	Presumptive identification is <i>M. pneumoniae</i>	Presumptive identification is a species other than <i>M. pneumoniae</i>
1.	A section of agar with 20 or more colonies is removed from the plate.	
2.	Place agar block in screw-cap tube containing 3.0 ml PPLO broth	Place agar block in screw-cap tube containing 3.0 ml 0.85% NaCl solution.
3.	Shake vigorously on electric Vortex mixer for 10-20 sec. to dislodge organisms from agar (Fig. 25).	
4.	Incubate tube at 37 C for 4 days.	Transfer 0.3 ml saline suspension to 100×15 mm agar plate. Spread with bent glass rod. (Fig. 26).
5.	Transfer 0.3 ml broth culture to 100×15 mm agar plate, spread with bent glass rod.	

STEP 5. DRYING

The next step, drying the agar medium before applying the discs, is important. Dehydration is accomplished by placing the plates face up in a 37 C incubator for 15 to 20 min with lids propped on one side to allow escape of moisture. A good test for dryness is to hold the plate in an inclined position watching for moisture droplets to flow downward on the agar surface. Agar medium properly dried for the test is free of such excess but is moist enough to still glisten in reflected light.

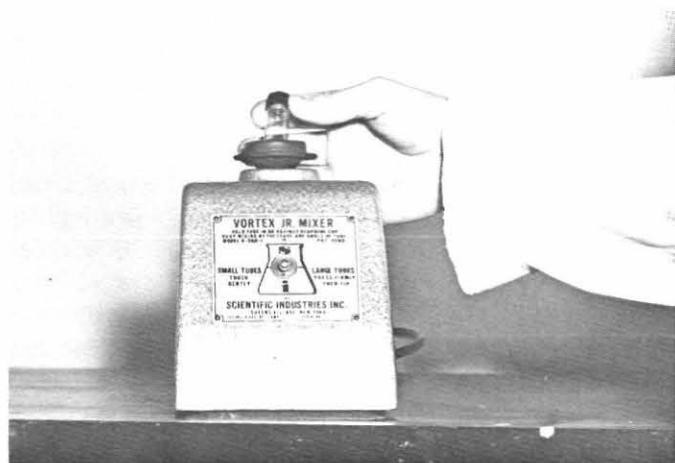


Fig. 25. Treating an unknown culture by Vortex mixer to obtain a good inoculum for the disc test.

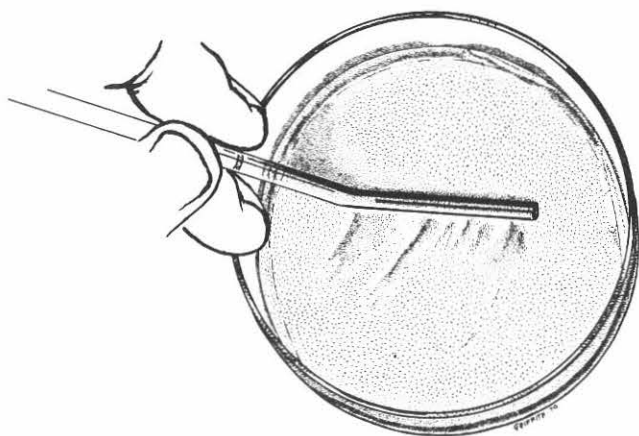


Fig. 26. Spreading inoculum with bent glass rod.

STEP 6. PREPARATION, CHARACTERISTICS, AND USE OF PAPER DISCS

Paper discs for the sero-identification test may be prepared from filter paper. An ordinary office punching device that cuts 6 mm holes, in pairs, provides discs for many tests with a minimum of time and effort. Discs so obtained are easily sterilized and dried in screw cap tubes by autoclave. Just before use it is convenient to pour them into a sterile Petri plate for selection with thumb forceps. Hand punched discs are difficult to separate but a vigorous shaking of the Petri plate will assist in breaking them apart. Each 6 mm disc prepared from filter paper of medium porosity will absorb approximately 0.005 ml of serum (0.1 ml serum saturates 15 discs).

Commercially available, blank, sterile, paper discs for sensitivity determinations offer the advantage of being easier to handle. They are useful for

quantitative work because each disc absorbs 0.025 ml serum which can be loaded with a calibrated microtiter loop. Whether prepared from filter paper or obtained commercially, discs that are wetted by hand-dipping usually are overly saturated. The excess can be removed by touching the disc to a piece of filter paper in a covered Petri dish just before placing it on the agar.

Since growth-inhibiting antibody withstands freezing and thawing stresses, one may find it more convenient to saturate the discs in advance for storage in the -20°C freezer until needed. A useful kit can be prepared from a piece of wood and 1 dram screw-cap vials (Fig. 27). A piece of soft wood



Fig. 27. A device for storing individually saturated paper discs in the freezer.

$1'' \times 2'' \times 10''$ is drilled with 6 wells $\frac{1}{2}''$ deep and $\frac{3}{4}''$ in diameter. The edges of the upper surface are beveled. The vials are labeled to show species, date prepared, and the approximate inhibitory zone to expect in millimeters. The entire kit can be placed at -20°C and withdrawn for thawing as needed.

These descriptions have emphasized the use of single paper discs which have inherent disadvantages. Each disc becomes a separate operation. Then, its location in the plate must be indicated by a pencil marking. This label can be avoided by using 8-tipped discs presently available on the market (Fig. 28). They can be obtained with the tips labeled by

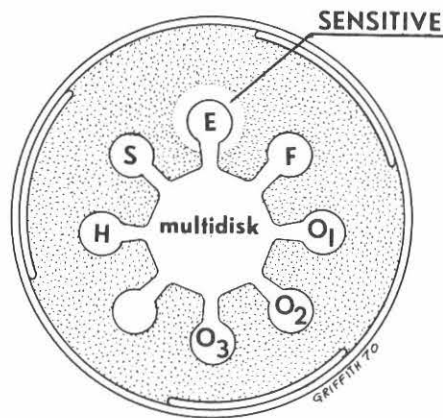


Fig. 28. An 8-tipped disc for serological identification of mycoplasma species.

the manufacturer at little extra cost. An 8-tipped disc is preferable to 6 tips because extra blank tips can be used for (a) control on paper chemicals that might be inhibitory, and (b) for testing additional sera.

The labeled discs are packaged in screw-cap jars, 50 discs/jar, and are impregnated with antiserum under a hood. By holding packets of 12–15 discs with 2 sterile forceps, tips are touched to the surface of corresponding antisera in wells of a sterilized porcelain spot plate or in Stender jars. It is important to pinch the tips tightly with one of the forceps when they are touched to the serum. When the 6 tips have been saturated, they are placed in sterile Petri dishes until all 50 discs from the manufacturer's screw-cap jar have been completed. Then all are replaced in the original container and stored frozen at -20°C until needed. Although more refined techniques may seem desirable the above procedure enables one to process as many as 1,000 discs in 2 to 3 hr.

Species Identification of Mycoplasma Colonies by Immunofluorescence

In 1967, Del Giudice, Robillard, and Carski described a fluorescent antibody method for rapid identification of mycoplasma colonies growing on agar (15). The technique, which can be applied to primary isolation plates, distinguishes not only normal mycoplasmas, but also those which grow into atypical colonies. The fluorescent antibody method is particularly valuable for analyzing mixtures of serotypes on the same plate. The procedures below are those described by Del Giudice and colleagues.

STEP 1. PREPARATION OF ANTISERA

Horses, mules, or goats are immunized for prolonged periods of time, i.e., 4 to 11 months. They are injected with 100x concentrates from 500 ml broth cultures and are injected with 10^7 to 10^{10} organisms/dose. In order to avoid nonspecific fluorescence, the broth used for growth is enriched with serum derived from the same animal scheduled to be immunized. After a primary course of injections the animals are rested 2–6 months and then given booster injections of 1 or more doses of antigen.

STEP 2. CONJUGATING THE ANTIBODY

The globulin is obtained from the antiserum by precipitating with half-saturated neutral ammonium sulfate in an ice bath. The precipitate is taken up in water and dialyzed against saline to eliminate

sulfate. Labeling is performed with fluorescein isothiocyanate of high chromatographic purity. One part fluorescein to 70 parts protein in a solution buffered at pH 8.8 is allowed to react overnight at 4 to 10 $^{\circ}\text{C}$. Free, or unbound fluorescein is removed by filtration through a Sephadex G-25 column adjusted to pH 7.2–7.4 with 0.01 M phosphate buffered saline. The conjugate is finally dialyzed overnight against 0.01 M phosphate buffered saline at pH 7.2–7.4.

STEP 3. STAINING THE SPECIMEN

The medium used is that of the standard formula described on page 14, except that 2% yeast dialysate is substituted for 10% crude yeast extract. Polystyrene plates, 60×15 mm, are used. When the colonies become microscopically detectable, the agar surface is first flooded with 5 ml pH 7.2 buffered saline, and held at room temperature for 20 min. The optimal dilution of conjugate is determined beforehand by titration. Staining titers range from 1:20 to 1:320. The working dilution is the highest dilution that produces a 3+ or 4+ staining reaction. After 30 min incubation, the conjugate is decanted and the agar surface is rinsed 3 times by flooding with buffered saline.

STEP 4. FLUORESCENCE MICROSCOPY

Del Giudice and associates examined the stained colonies with a Zeiss GFL 658 binocular microscope equipped with an Osram HBO 200 mercury vapor lamp. In order to avoid transmission of ultraviolet light through agar and plastic plate the microscope was fitted with an incident illumination attachment. A BG 12 exciter filter with a No. 47 barrier filter and bright field condenser were also applied.

The colonies remain intact through the staining and washing procedures because of their tenacious attachment in the agar. When properly stained, the colonies show yellow-green fluorescence while the agar and heterologous colonies show bluish autofluorescence. High magnification shows that the stain is adsorbed by the outer membranes of large bodies. Colonies 2 to 3 weeks old may show diminished staining reactions within the central core, while younger colonies give superior staining reactions.

Plates inoculated with tissue culture material show granular interstitial fluorescence not in the form of typical colonies. Throat washings inoculated on mycoplasma plates and examined by fluorescence microscopy show a close association of mycoplasmas and epithelial cells.

Identification of T-strain Mycoplasmas

Microscopic Recognition

T-strains of mycoplasmas produce colonies which are reknowned for small size (Figs. 7, 8). They usually attain diameters of 14–24 μ , rarely exceed 50 μ , but decrease in size with increased crowding. While the surface zone, or peripheral halo, is usually lacking in primary T-strain cultures, very thin surface zones may nevertheless appear (see Fig. 7). The examination of shape and structure of the colony should be made under 100x magnification using an agar block mount overlaid with Dienes stained coverslip.

On ordinary T-strain medium, colonies usually become greenish-blue with Dienes stain, whereas large colony mycoplasmas stain more deeply royal blue (16). A typical T-colony has an irregular outline and ramifies in various directions beneath the agar surface. Under the electron microscope individual organisms show up as coccoid shaped (1). Both agar and broth cultures show spherical particles bound in clumps by a gelatinous matrix (see Fig. 8). The differentiation of T-strain colonies from classical species is a simple procedure on the A-6 medium of Shepard (see page 17). The T-strain colonies develop a dark, golden brown color due to the action of manganous sulfate. An agar section is placed on a glass slide and a coverslip with dried Dienes stain is placed on the surface. The smaller golden brown T-strain colonies remain golden brown whereas the larger colonies of classical species, such as *M. hominis*, become blue.

Urease Test for T-strain Identification

T-strains of mycoplasma hydrolyze urea with production of ammonia and a concomitant shift from an acid to an alkaline reaction (17,18). While the non-fermentative, classical species utilize arginine, neither fermentative nor non-fermentative large-colony species metabolize urea. Hence, the hydrolysis of urea may be utilized as a test for T-strain identification. The objective of the test is to detect the upward pH shift as urea is split and ammonia liberated. This may be accomplished by growing the specimen in liquid urease test medium U9 (page 17). The urease test may be performed on solid medium or in broth. The incorporation of urea and phenol red in liquid medium for primary isolation provides one method for presumptive identification. Ford and MacDonald as well as Shepard and Lunceford observed that urea concentrations of 0.05% and 0.06% were near optimum for fluid

cultures (17, 18). Taylor-Robinson and colleagues utilized PPLO broth and 0.1% urea for cultivating bovine T-strains (19, 20). Williams and Taylor-Robinson used 0.1% and 1.0% concentrations of urea in agar medium and noted the production of a brownish coloring substance by T-strains (21).

Appearance of Mycoplasmas Under the Electron Microscope

Under the electron microscope, the mycoplasmas of human origin have been observed to possess characteristic shapes. The electron photomicrographs of Clyde and Kim (2) shown in Fig. 29 reveals the structural units of 6 species of human origin as they appeared from log phase broth cultures. *Mycoplasma salivarium* (A) consisted of spherical elements of 300 m μ and rod shaped units 180 m μ thick by 0.6–1 μ long. Structures from *M.*

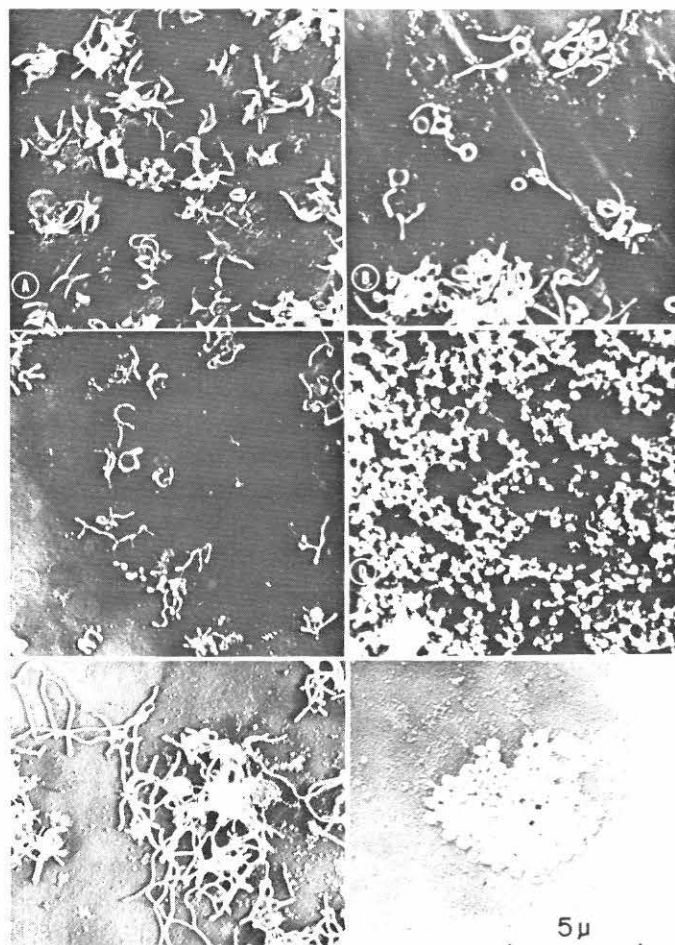


Fig. 29. Sediments from log phase broth cultures of mycoplasma species of human origin under electron microscope. A formalin vapor fixation-drying technic was used. (A) *M. salivarium*, (B) *M. hominis*; (C) *M. fermentans*; (D) *M. pneumoniae*; (E) *M. orale* type 1; (F) T-strain mycoplasma. Reproduced with permission of W. A. Clyde, Jr., and editors of "Annals New York Academy of Sciences."

CHARACTERISTICS OF MYCOPLASMAS OF HUMAN ORIGIN*

Reactions or characteristics	M. pneumoniae	M. hominis I	M. salivarium	M. orale 1	M. orale 2	M. fermentans	T-strain
Tetrazolium reduced aerobically	+	—	—	—	—	—	?
Growth on 0.002% methylene blue agar	+	—	—	—	—	—	?
Dextrose utilized without gas	+	—	—	—	—	+	—
Arginine hydrolyzed with production of NH ₃	—	+	+	+	+	+	—
Methylene blue reduced	+	±	±	?	?	+	?
Urea hydrolyzed	—	—	—	—	—	—	+
Resistant to 1:2000 thallium acetate	+	+	+	+	+	+	—
Turbidity in broth (G=granular; D=diffuse; F=faint.)	G	F	D	D	D	D	D
"Fried egg" colonies typically produced	—	+	+	+	+	+	—
Spots or film on normal horse serum agar	—	—	+	—	—	+	—
Hemolysis of erythrocytes							
of sheep	β	γ	α	γ	?	α	α'
guinea pig	β	α'γ	α	γ	α	α	β
horse	αβ	α'γ	α'	γ	?	α	γ
human O	α	γ	γ	γ	?	α	α'
Hemagglutination of guinea pig erythrocytes	+	—	—	—	—	—	—

*FOR ADDITIONAL INFORMATION SEE REF. 1

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hominis (B) were almost twice as big. Twisted and angular filaments 120 m μ by 1.5 μ were seen from *M. fermentans* (C). The morphology of *M. pneumoniae* (D) was staphylococcal with elements of 250–300 m μ . A marked filamentous configuration distinguished *M. orale* type 1 (E) with elements 120 m μ across and up to 8 μ long. T-strain mycoplasma showed clusters of spherical bodies (F) resembling *M. pneumoniae*. These findings suggested division into 3 groups; (1) filamentous forms are marked (*M. orale*), (2) moderate (*M. salivarium*, *M. fermentans*), (3) or scant or absent (*M. pneumoniae*, T-strains).

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Antibodies directed toward the mycoplasmas have been measured by a variety of quantitative and qualitative techniques. A fluorescent antibody staining method, developed by Liu, was originally used for the study of Eaton agent infections (1). While this method was reliable and sensitive, it was technically cumbersome since frozen sections of infected chick embryo lungs were utilized for the antigen. The simplest and most practical method for detecting *M. pneumoniae* antibody is by complement fixation (CF). The CF fraction of *M. pneumoniae* is a heat stable lipid extractable with organic solvents. The antigen for this test is derived from (a) broth grown organisms, or (b) organisms grown by attachment to glass.

Preparation of Phenolized *M. Pneumoniae* Complement-Fixing Antigen From Broth Cultures

The phenol method of Nigg and Hilleman was adapted by Chanock and associates to produce *M. pneumoniae* CF antigen from fluid cultures (2, 3). The FH (ATCC #15531) and GL-M52 (ATCC #15293) strains which ordinarily yield 10^5 – 10^6 colonies/ml have been reliable strains for antigen production in the author's laboratory.

STEP 1. THE BROTH CULTURE

Fluid medium is prepared by the usual formula (p. 14). One may estimate that each liter of fluid medium will result in 20 ml of antigen having a titer of 1:32. A supplement of gamma globulin-free horse serum should be used in preference to crude horse serum because the anticomplementary activity of the final material is reduced. Either 1 liter of broth in a 2-liter flask or 2 liters of broth in a 4-liter flask are used for growing the culture.

STEP 2. INOCULATING THE FLASK

The liquid medium for growing the organism is inoculated from a pilot flask, or tube, containing a 7-day growth of *M. pneumoniae*. Growth in the pilot flask is initiated with either agar blocks or a liquid stock culture. The volume of inoculum sufficient for promoting growth under ordinary conditions is 5 ml for each liter of final culture.

After inoculation, the flasks are swirled by hand and placed at 36–37 C. It has been reported that "shaker" cultures are productive of higher colony counts (4). However, information is not available on the titers of CF antigen obtained from such growth. Growth will be facilitated, nevertheless, by

rotating the flask by hand at least once each day during the period of incubation. Incubation is continued 9 to 13 days before the culture is subjected to centrifugation.

STEP 3. CENTRIFUGATION

The small particle size of the CF antigen requires high-speed centrifugation for better harvest. A Spinco model L ultracentrifuge revolving at 30,000 rpm for 30 min gives excellent results, but the small volume output means that much time will be required to process large volumes of material. The refrigerated Serval Superspeed model RC-2 with a continuous flow device, spinning at 16,000 rpm can process up to 6 liters of broth culture in 8 hours with the SS-34 head (radius=4.25 inches). In the author's experience, the final titer of antigen obtained with RC-2 material has not differed from titers obtained with the ultracentrifuge. Normally, the sediment is gray to black in color. Any large mass of white material should be regarded with suspicion, and may be traced to precipitates in the horse serum.

STEP 4. RESUSPENSION OF PELLET

Following removal from the centrifuge, the tubes are decanted and inverted over paper towels for several minutes to allow drainage. Using a 10 ml pipette the sediment is taken up in phenolized veronal buffer (see p. 12). Twenty milliliters of buffered saline is used to suspend the sediment collected from each liter of culture. Storage in a screw-cap bottle will be convenient for the serologists. Aggregates of organisms in the final suspension do not break down well from hand shaking. If the material is shaken with glass beads on a mechanical shaking device the gummy clumps disappear and the suspension becomes homogenous.

STEP 5. REDUCING ANTICOMPLEMENTARY EFFECT

The antigen prepared as outlined above may be anticomplementary. If the anticomplementary titer is 1:4 or greater, the screw-cap bottle containing the antigen is placed in a boiling water bath for 15 min after freeing the cap. It will be observed that this treatment effectively reduces, or removes entirely, the anticomplementary effects with no apparent loss of antigen content.

NOTE ON STORAGE OF PHENOLIZED ANTIGEN

It is unnecessary to store the phenolized antigen in the frozen state. The presence of phenol permits

storage at room temperature for periods of at least 6 months without contamination or loss of titer.

Note: The CF fraction of *M. pneumoniae* grown in broth may be extracted with organic solvents according to the method of Kenny and Grayston (5). The purified material is free of anticomplementary activity and is thought to react with greater specificity. See page 43 for method.

Preparation of Complement-Fixing Antigen From *M. Pneumoniae* Grown on Glass Surfaces

Colonies of *M. pneumoniae* can be cultivated in masses on the surface of glass (6). The advantages of growing the organism on a glass surface are that they can be easily washed, harvested, and concentrated without centrifugation (Fig. 30). The antigen so obtained is of reduced anticomplementary activity, is highly specific, and has a very high titer. The organisms for CF antigen are grown in 2-liter Povitsky bottles.

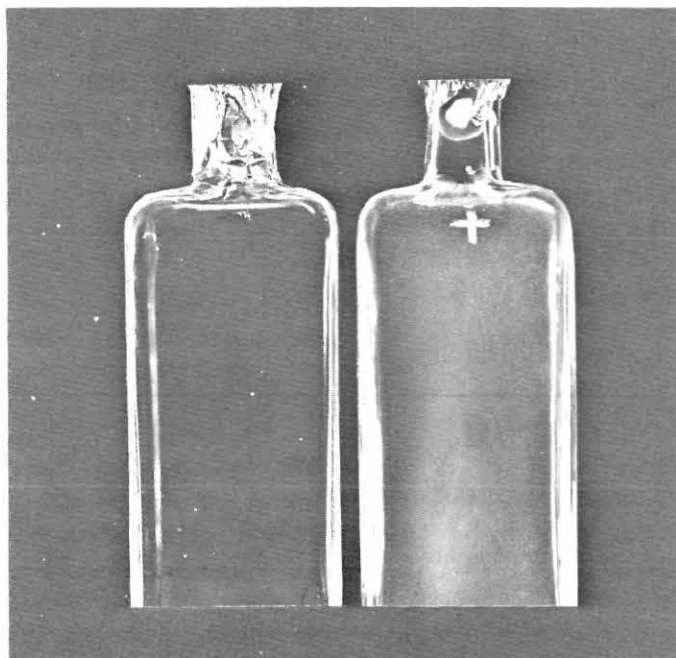


Fig. 30. *Mycoplasma pneumoniae* colonies adhering to glass surface (right) of a two-liter Povitsky bottle.

STEP 1. BROTH MEDIUM

Prepare enough HEPES buffered liquid medium to provide 100 ml for each 2 L Povitsky bottle. The HEPES broth medium, slightly modified from that of Conant *et al* (7), is described on page 16.

STEP 2. INOCULATION

The medium should be heavily inoculated. Somerson, *et al* used 10^9 colony forming units to inoculate

500 ml of broth. This would approximate a 2.0 ml volume of a normal 3-day broth culture. The FH strain (ATCC 15531) and M-52 strain (ATCC 15293) of *M. pneumoniae* grow well on glass. There is ample reason to believe that all strains of *M. pneumoniae* will adhere to glass if grown under the specified conditions.

STEP 3. INCUBATION

The bottle(s) are placed in a 36–37 C incubator. Somerson, *et al* found that 6 days of incubation are near ideal. The incubation period undoubtedly will vary around the 6-day period and the decision to harvest will depend upon the judgment of the worker. Conant, *et al* reported that 4 days with their medium was sufficient. A color change from red to yellow provides the clue of a pH drop and approaching death of the culture.

STEP 4. WASHING AND HARVESTING

After decanting the broth medium from the bottle, carefully wash the sheet of growth 3 times with pH 7.3 phosphate-buffered saline (see p. 13). For the final suspension, add 20 ml phenolized veronal buffered saline, if the phenolized CF antigen is desired. Scrape the growth from the glass surface with a rubber policeman on a glass rod bent 90°. The bent rod permits scraping the area adjacent to the neck of the bottle which is inaccessible if a straight handle is used. The resulting turbid suspension is transferred to a screw-cap bottle and stored on the shelf at room temperature to await testing. An alternate method of removing the organisms from the glass is to treat with 2.5% trypsin (6).

Extracting *M. Pneumoniae* Complement-Fixing Antigen From Organisms Grown On Glass Using Organic Solvents

The anticomplementary activity of *M. pneumoniae* organisms presents a hindrance to the performance of complement fixation tests. The anticomplementary activity of *M. pneumoniae* antigen can be removed by heating (see p. 41). It can also be reduced by extraction with organic solvents, a method recommended by Kenny and Grayston (5). The active fraction, a lipid, is soluble in ethanol, ether, chloroform, and chloroform-methanol. Utilizing organisms grown on glass, in the medium of Conant *et al*, (see p. 16) the harvesting and extraction can be performed as follows:

STEP 1. HARVESTING ORGANISMS

When a lowering of the pH occurs (4 days), discard the 100 ml HEPES broth from the bottle. Add

10 ml phosphate buffered saline pH 7.3 and wash the layer of colonies clinging to glass surface. Discard and repeat washing procedure for a total of 3 washings. Add 10 ml phosphate buffer and remove growth with a rubber policeman. Decant, and repeat the removal procedure 2 more times and combine all 3 to obtain maximum yield. Pool the 3 batches.

STEP 2. CENTRIFUGATION

The suspension from step 1 is subjected to centrifugation. A Servall superspeed centrifuge with an SS-1 head spinning at 10,000 rpm for 1 hr packs a firm pellet.

STEP 3. EXTRACTING LIPID ANTIGEN

The complement-fixing lipid antigen of *M. pneumoniae* is obtained by means of a chloroform-methanol-aqueous-KCl partition. Decant the supernatant buffered saline from the pellet obtained from centrifugation. For each Povitsky bottle that formed the pool add 1 ml phosphate buffered saline to the organisms. Assuming 5 bottles were cultivated, take up resulting pellet with 5 ml phosphate buffered saline. Transfer this 5 ml suspension to a separatory funnel and add 50 ml methanol. Shake well. Then add 100 ml of chloroform and again shake the funnel. To the chloroform-methanol add 37.5 ml of 0.1 M aqueous KCl and shake vigorously. Place the mixture in the refrigerator overnight or in an ice bath for 1 hr to obtain phase separation. Two clean phases result, i.e., a chloroform phase and a methanol phase. The antigen is in the chloroform phase at the bottom. Evaporate the chloroform phase and take up the residue with 5 ml of phosphate buffered saline. Shake with glass beads to obtain dispersion. The antigen so obtained should contain 128-256 units/ml of antigen against 4 units of antibody.

Titrating *M. Pneumoniae* Complement-Fixing Antibodies

Of the tests available for measuring *M. pneumoniae* antibodies the complement fixation test is the most suitable for the average laboratory. These antibodies can be measured in test tubes by the method of Kolmer (8). Either the 37 C one-hour-of-incubation test or the overnight-incubation-in-the-cold system may be used for *M. pneumoniae* CF antigens. It has been customary to employ 2 units of complement, 2 units of hemolysin, and 2 to 4 units of antigen in the test system. Complement, hemolysin, *M. pneumoniae* CF antigen, and *M. pneumoniae* standard antisera are all available from commercial sources (see supply table). The use of mi-

cro-titer equipment is to be encouraged because of titration and economy of materials (Fig. 31). References on the use of these tools should be consulted if the worker is unfamiliar with their intricacies (9, 10).

The procedure for performing the microtiter test for *M. pneumoniae* CF antibodies is outlined below. Before final titration of patient sera it is essential to standardize hemolysin, complement and antigen.

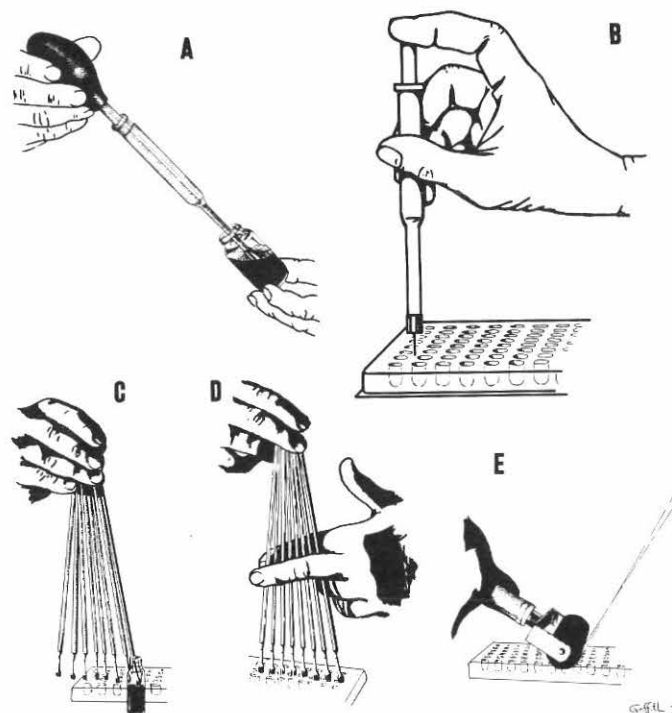


Fig. 31. Microtiter techniques: Filling pipette with reagent (A), adding drops to microtiter plate (B), loading diluters with specimen (C), making multiple serial dilutions simultaneously (D), and taping the plate (E).

STEP 1. STANDARDIZING HEMOLYSIN

The dilution of hemolysin (rabbit serum containing antibody for sheep erythrocytes) needed to sensitize sheep RBC for the final test is determined as follows: Prepare a 1:5 dilution of 1:100 hemolysin (see p. 13). The final dilution is now 1:500. Add one 0.025 ml drop of cold veronal buffered saline (VBS) to 7 wells in 2 rows of a microtiter plate with "U" shaped wells. Then add 1 extra drop to the first well of the 2nd row. Using a primed 0.025 ml loop diluter, serially transfer the 1:500 hemolysin through 6 wells. Add 2 drops of saline to each of these 6 wells and 4 drops to the 7th well. Next, add 1 drop 2% sheep red cell suspension to all wells. Mix by tapping the plate. The plate is then incubated

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for 30 min at 37 C. Following the 30 min incubation period, 2 drops of complement, diluted 1:60, are added to all but the 7th well. The plate is then covered with tape, mixed well, and incubated at 37 C for another 30 min period. The plate is then spun in a centrifuge equipped with special carriers at 1500 rpm for 5 minutes.*

Reading the test: The highest dilution that causes hemolysis of all erythrocytes in the well is 1 hemolytic unit. In the final test 2 units are used. Hence, if the hemolytic end point occurs in the 1:8000 well, the hemolysin should be diluted 1:4000 for the final test. The stock solution, it should be remembered, is already diluted 1:100.

Dilutions in the hemolysin titration

	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
A	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000	Control
B	1:1500	1:3000	1:6000	1:12,000	1:24,000	1:48,000	Control

Sensitization of 2% sheep RBC suspension: An equal volume of properly diluted hemolysin and 2% sheep RBC are mixed and incubated in a 37 C water bath for 10 min. These RBC, now sensitized with sheep RBC antibody, are ready for the remaining steps of the test.

STEP 2. STANDARDIZING COMPLEMENT

(Pooled, normal guinea pig serum)

Two exact units of complement are used in the final test. The dilution of guinea pig serum containing these 2 units is determined as follows: Prepare a series of six dilutions of complement, 1 ml each, as follows: 1:30, 1:40, 1:50, 1:60, 1:70 and 1:80. Keep these dilutions cold to avoid loss of activity. For each dilution of complement prepare a 4-well test as indicated below:

Reagent	<u>1st well</u>	<u>2nd well</u>	<u>3rd well</u>	<u>4th well</u>
VBS	0.025 ml	0.0375 ml	0.05 ml	0.0625 ml
Antigen**	0.025	0.025	0.025	0.025
Complement 0.05		0.0375	0.025	0.0125†
		<u>Cell Control</u>	<u>Hemolytic Control</u>	
VBS		0.1 ml	0.05 ml	
Complement		—	0.05 ml	

When all reagents have been added to the wells the plate is incubated for 1 hr at 37 C. Then, 0.05 ml sensitized RBC is added (2 drops from 0.25 ml dropper). The plate is then incubated for 30 additional min at 37 C.

* Carriers for holding microtiter plates are available from Cooke Engineering Co., Alexandria, Virginia.

** Use 4 units antigen/0.025 ml

† 0.0125 ml quantities are added with a 0.1 ml pipette

Reading the test: The dilution of complement that most nearly gives the following pattern in the above test is the proper dilution to use:

	<u>1st well</u>	<u>2nd well</u>	<u>3rd well</u>	<u>4th well</u>
Reading	0	0	0	4
	(Complete Hemolysis)			(No hemolysis)
No. Units	2	1.5	1	0.5

If hemolysis occurs in the 4th well there is an excess of complement. If cells are present in well No. 3 the system has less than 2 exact units. It is wiser to use slightly less than 2 exact units of complement rather than an excess.

STEP 3. STANDARDIZING *M. pneumoniae* ANTIGEN BY STRAIGHT LINE TITRATION

The dilution of antigen to use in the final test is determined as follows: Omitting the first wells, add 0.025 ml VBS diluent out to the 11th well using 2 rows in the plate. Drop 0.05 ml undiluted antigen in each of the first wells and prepare serial 2-fold dilutions with a 0.025 ml loop diluter. Prepare a 3rd and 4th row of wells in the same way but use an antigen of known titer. Add one 0.025 ml drop of inactivated immune serum diluted to contain 4 units antibody to each well in row No. 1. To each well in row No. 2 add 1 drop 0.025 ml VBS. The purpose of the 2nd row is to determine the anticomplementary titer of the antigen. Add 2 drops, or 0.05 ml, of properly diluted complement to all wells except the control. The test is controlled according to the outline below:

CONTROLS FOR THE ANTIGEN

1. Complement control: Using 4 wells of the plate add the reagents indicated below:

Reagent	<u>1st well</u>	<u>2nd well</u>	<u>3rd well</u>	<u>4th well</u>
VBS	0.025	0.0375	0.05	0.0625
Antigen*	0.025	0.025	0.025	0.025
Complement	0.05	0.0375	0.025	0.0125
Units Complement	2.0	1.5	1.0	0.5

2. Hemolytic control: To 1 well add 0.05 ml VBS diluent and 0.05 ml complement.
3. Antigen control:** To assure that the antigen does not hemolyze RBC or

* Unless antigen is known not be anticomplementary, replace with VBS.

** Antigen control is run with the dilution that is used in the test; and, to observe anticomplementary effect, at one-tenth this dilution. This antigen should be a known or previously standardized antigen.

is not anticomplementary, add 0.075 ml diluent and 0.025 ml antigen to 1 well.

4. Cell control: Add 0.1 ml diluent to 1 well of the plate. The cell control is included for assurance that the diluent does not lyse the RBC.

When all reagents have been incorporated in the test incubate the plate for 1 hr at 37 C. Then add 2 drops (0.05 ml) sensitized sheep RBC suspension to all wells and mix. Tape the plates and incubate again at 37 C until the 4-well complement control reaches its reading pattern (see complement titration). The plates are then subjected to centrifugation for 5 min at 1500 rpm.

Reading the test: The highest dilution of antigen which is completely free of hemolysis is equal to 1 unit. From 2 to 4 units are customarily used in the final test. If the endpoint is 1:128 then 2 units would be contained in a 1:64 dilution and 4 units in a 1:32 dilution.

STEP 4. DETERMINING THE COMPLEMENT-FIXING ANTIBODY CONTENT OF SERUM

(Final Test)

When hemolysin, antigen and complement have been standardized the antibody content of a serum can be determined. The microtiter plates for this titration should have wells of the "U" shape. The method of incubating serum, antigen, and complement is optional, i.e., either for 1 hr at 37 C or overnight at 4 C. In many systems the fixation of complement is best accomplished by incubating overnight in the cold.

Preparation of serum dilution: Sera are heated at 56 C for 30 min without dilution. They should be well shaken before progressing with the test. Add 1 drop of diluent to wells of the plate using a 0.025 ml dropper, but do not add drops to last wells in rows which are reserved for controls. Then add 2 more drops to the first wells in the rows for a total of 3 drops. Dip into the previously inactivated serum with a 0.025 ml loop diluter and transfer the loaded loop to the first well. Rotate the stylus at least 4 times to insure mixing. The dilution in the 1st well is now 1:4. Carry out serial dilutions to all but the last 2 wells. The second to last well is to be used for the serum control to determine its anticomplementary activity. Drain the loop on blotter or gauze. Reload loop from first well and transfer to second from last well. This well now contains a 1:8 dilution for serum anticomplementary control.

Addition of antigen: The antigen, properly diluted as determined from previous titration, is added next. Drop 0.025 ml into all wells except the serum controls in the 11th wells.

Addition of complement: Complement is the 3rd reagent added to the wells. Drop 0.05 ml (2 drops) containing 2 exact units into all wells including serum control. Shake the plates lightly to mix.

Preparing controls: Add 1 drop (0.025 ml) diluent to the serum control well (2nd from last in row) to bring to equivalent volume. Four wells in the last row are used for RBC control, hemolytic control antigen control, and antigen anticomplementary control. The RBC control is included to insure that no hemolysis occurs in the presence of diluent. The hemolytic control insures that complete lysis occurs. The antigen control is used to prove that the antigen by itself is not hemolytic. The antigen anticomplementary control shows whether or not the antigen itself fixes complement. These wells are prepared as follows:

Reagent	Well Number			
	1	2	3	4
	Cell control	Hemolytic control	Antigen control	Antigen anticomp. control
Diluent	0.1 ml	0.05 ml	0.075 ml	0.025 ml
Antigen	—	—	0.025	0.025
Complement	—	0.05	—	0.05

A four-well complement control is used with each plate to insure that two exact units of complement were utilized in the test. This is necessary where a large number of plates (tests) are run. The time lag in adding the sensitized RBC from plate number 1 through plate number 20 is sufficiently long that such controls are essential. This is not as critical when 1-3 plates are used in a run, but is recommended even in small runs. The reagents for making this measurement are prepared as follows:

Reagent	Well Number			
	1	2	3	4
Diluent	0.025 ml	0.0375 ml	0.05 ml	0.0625 ml
Antigen	0.025	0.025	0.025	0.025
Complement	0.05	0.0375	0.025	0.0125
Units of Complement	2.0	1.5	1.0	0.5

In adding the above volumes it is necessary to add 0.0125 ml to certain wells to obtain the final volume. Since this cannot be accomplished with usual droppers the 0.0125 volume is added with a 0.1 ml pipette.

It is next essential to include positive and negative serum controls of known titer. These sera are treated exactly as the unknown test sera.

Incubation: When all of the above steps have been completed stack and cover the plates to prevent dehydration. Place stacks in refrigerator operating at 4–6 C for 18 hrs or overnight. Following this period of incubation remove the plates and allow to warm at room temperature for 10 min. Add 2 drops (0.05 ml) sensitized RBC to every well in the plate. Cover plates with sealing tape and mix vigorously. Incubate at 37 C until complement control has attained the correct degree of hemolysis i.e., complete hemolysis in wells No. 1, 2, 3, and a 4+, or no hemolysis, in well No. 4.

Remove the plates from the incubator and spin in the centrifuge for 5 min at 1500 rpm. An alternate method is to place the plates at 4 C until the cells settle to the bottom of the wells.

Reading and interpretation: The endpoint of each serum is considered the highest dilution giving complete fixation of complement (no trace of hemolysis). Serum controls should show no fixation. The RBC control, and antigen control wells should likewise show no fixation. The hemolytic control and antigen anticomplementary controls should show complete lysis. Partial hemolysis in wells 1, 2, and 3 signifies a shortage of complement activity. If hemolysis is present in well No. 4 then an excess of complement is present. The positive and negative control sera should show end-points not to exceed ± 1 dilution from the true titer.

The Tetrazolium Reduction Inhibition (TRI) Test

The titration of growth inhibiting antibody using the suppression of colonial growth as the marker of antibody endpoints has been attempted by various workers. The techniques so far attempted have been laborious, inexact, and quantitatively insensitive. For this reason, other tests have been developed which measure the ability of a serum to suppress a metabolic reaction by a mycoplasma. For example, *M. pneumoniae* is an active reducer of 2,3,5-triphenyl-2H-tetrazolium chloride under aerobic conditions and will do so either on agar, in broth, or in a microtiter plate. The colorless tetrazolium salt, when reduced, changes to a reddish hue, and then to crystals of dark red formazan. In the presence of antibody, the reduction of tetrazolium is inhibited, and the color change fails to take place. Utilizing this principle Senterfit and Jensen described methods for determining tetrazolium reduction inhibition titers of animal and human sera (11).

STEP 1. PREPARE TRI MEDIUM FOR DILUTING FLUID

The diluting fluid used for the TRI test is essentially the medium described on page 16. Seven parts PPLO broth, 1 part 25% yeast extract, 2 parts horse serum-penicillin, and 500 ppm thallium acetate are supplemented with 1% dextrose. To this medium enough stock solutions of 2,3,5-triphenyl-2H-tetrazolium chloride solution is added to give a final concentration of 0.05%. Just before use 5.0 ml normal guinea pig serum previously inactivated at 56 C for 30 min, added to 95 ml of above broth, completes the TRI liquid medium.

STEP 2. PREPARING THE INOCULUM

In order to prepare a viable inoculum for the test, inoculate standard broth medium (not TRI) with *M. pneumoniae*. Others have noted that either the FH strain or the PI-1428 strain of *M. pneumoniae* gave equivalent results as long as the standard inoculum used in the test was 1 TRI unit. *One TRI unit is defined as the greatest dilution of organisms which causes a color change after a standard incubation period of 6 days.* When the culture to be used as inoculum has incubated 5 to 7 days store aliquots at -70 C in screw-cap vials for subsequent titration and use in the antibody titrations.

STEP 3. STANDARDIZING THE INOCULUM

To determine the dilution of organisms to use in the antibody titrations thaw a vial from the frozen state and titrate in microtiter plates as follows:

- (a) Add 0.025 ml standard TRI broth with guinea pig serum to all but the 1st well.
- (b) Add 0.05 ml undiluted, thawed culture to 1st well.
- (c) Serially transfer 0.025 ml from 1st well.
- (d) Add 0.175 ml of TRI medium with guinea pig serum to each well. Dilution of *M. pneumoniae* in 1st well=1:8.
- (e) The plate is sealed and incubated at 37 C (in the dark to prevent photoreduction).

The endpoint is read after 6 days of incubation and is recorded as the greatest dilution showing color change (1 TRI unit). This endpoint typically is about 1:64 (well No. 4) and corresponds to approximately 10^5 CFU of *M. pneumoniae*.

STEP 4. CONTROLLING THE FINAL TEST

The controls on TRI antibody determinations may be included together in separate microtiter plate. The controls to use are as follows:

- (a) *Mycoplasma growth control*: One row of wells consists of a titration of the inoculum. The procedure is exactly as described in step 3 above. This titration is used as a guide, i.e., when the endpoint indicates that 1 TRI unit is present in the test (usually in 6 days) the serum endpoints are accepted as terminal.
- (b) *A positive serum control*: A serum of known TRI antibody content is titrated in each set of tests using a separate row of wells. For acceptable results the endpoint should not exceed ± 1 dilution (± 1 well) from the established titer.
- (c) *A negative serum control*: A serum of known negativity is titrated to reveal falsely positive TRI reactions. In the presence of dilutions of this serum tetrazolium should undergo reduction with no evidence of inhibition.

STEP 5. TITRATING TRI ANTIBODY CONTENT OF SERUM (Final Test)

The sera under examination are heated at 56 C for 30 min in the undiluted state. They are then titrated in microtiter plates according to the outline below.

- (a) Add 0.025 ml TRI medium to all but 1st wells.
- (b) Add 0.025 ml serum to both 1st and 2nd wells.
- (c) Serially dilute 0.025 ml forward from the 2nd well.
- (d) Add 0.175 ml appropriate dilution of *M. pneumoniae* inoculum to all wells (*M. pneumoniae* diluted in TRI medium). Serum dilution in 1st well=1:8.
- (e) Seal plates and incubate at 37 C.

STEP 6. READING AND INTERPRETATION OF TRI TEST

On the 6th day of incubation, the control plate is first examined for the mycoplasma growth control to show 1 TRI unit. The endpoint should be the same as that determined from previous standardization in step 3. For example, if the TRI unit was 1:64 in 6 days then the TRI unit should ideally occur in the 4th well (1:64) on the 6th day of the final test. The test should be read when this result is obtained. The serum antibody endpoints are the greatest dilutions of serum showing complete inhibition of tetrazolium reduction.

Fermentation Inhibition (FI) Test

Mycoplasma pneumoniae and *M. fermentans* convert dextrose to acid during their growth. In the presence of phenol red indicator the falling pH may be visualized by a color change from red to yellow. In the presence of growth-inhibiting antibody, multiplication and/or metabolism is ordinarily blocked and the red color of the medium remains stationary. Taylor-Robinson and his colleagues utilized this principle and devised a quantitative serological test for measuring growth-inhibiting antibody titer (12). They used microtiter plates and equipment without resorting to any special sterilization techniques. As deduced from their report, the modified scheme outlined below should simplify the performance of the FI test.

STEP 1. PREPARATION OF MEDIUM

The medium used throughout is the complete *M. pneumoniae* fluid medium to which is added 1% dextrose and 0.002% phenol red (0.1 ml of 2% solution per 100 ml medium). The final reaction should be pH 7.8. An exception is the horse serum supplement which should not be freed of gamma globulin. It is also important that the horse serum should be used unheated. A factor present in unheated guinea pig serum is known to enhance the activity of FI antibody for certain mycoplasmas and not others. When added to medium for FI antibody titrations, guinea pig serum has been used at a final concentration of 6%. Substituting fetal calf serum for horse serum also accelerates the reaction (13).

STEP 2. PREPARATION OF pH COLOR STANDARDS

The change of pH in the final test is measured visually by reference to color standards incubated in the same test. Divide broth medium containing phenol red into 14 aliquots of 20 ml each and transfer to screw-cap bottles. Using pH meter, 1 N HCl, and 1 N NaOH, standards are prepared in 0.2 pH unit increments ranging from pH 5.4 to 8.0. These standards are stored for use in step 5 of this procedure.

STEP 3. PREPARATION OF INOCULUM

While the number of organisms used in the test is not critical, the inoculum recommended is 10^3 color changing units (ccu). One ccu is defined as the highest dilution of mycoplasmas that will change standard broth medium with additives one-half pH unit over a specific time period. Inoculate 100 ml of complete broth medium containing 1% dextrose and 0.002% phenol red with a log phase suspension

of mycoplasma. When the pH changes from 7.8 to 7.3 (determined from standards, step 2) divide the culture into 1.0 ml amounts and store in screw-cap vials at -70°C .

STEP 4. STANDARDIZING THE INOCULUM

In order to achieve a standard inoculum of 10^3 ccu the culture needs to be titrated for dilution to be used. Thaw 1 ampule of the frozen inoculum from step 3. Prepare serial ten-fold dilutions in screw-cap tubes using complete medium (see Kelton's technique p. 58). From each dilution, 0.05 ml of broth medium is then added to each of these wells (total volume is 0.20 ml). The plates are sealed with special $3\frac{1}{2}$ in. tape or by covering the well with drops of mineral oil. Then they are incubated at 34°C without agitation until the pH color change no longer progresses downward. This period has been noted to last for 10 days. The highest dilution showing a pH change from the original 7.8 to 7.3 is the endpoint and contains 1 ccu. This determination is made by reference to a row of 14 wells containing 0.2 ml of each of the known color standard pH increments incubated in the same test.

STEP 5. CONTROLLING THE FINAL TEST

The controls on the final test are prepared together in a separate microtiter plate which is incubated together with the antibody titration plates. The controls are prepared as follows:

- (a) *pH color control*: Add 0.2 ml of each color control from step 2 to wells of the plate. These wells serve as pH indicator for reference with colors obtained in serum dilution tests. Contact with the plastic plate and tape may cause a slight shift in the color as incubation progresses.
- (b) *Mycoplasma growth control*: One well of the microtiter plate is reserved for observing growth and extent of color change produced by the organism in the medium being used in the absence of test serum. Add 0.15 ml (5 drops) medium with all supplements to 1 of the wells. Then add 0.05 ml (2 drops) mycoplasma suspension.
- (c) *Medium control*: Add to 1 well, 0.2 ml same medium with additives as used as diluent in test. Do not inoculate this well.
- (d) *Positive serum control*: A serum of known titer is included in the control plate. It is treated exactly as the test sera. For acceptable results the endpoint should not vary more than 1 dilution up or down from the known titer.
- (e) *Negative serum control*: A serum of known negativity is to be included in 1 row of the control plate to prove absence of falsely positive responses in the system. The serum is treated exactly as the test sera and positive control serum.

STEP 6. TITRATING MYCOPLASMA FI ANTIBODIES

Using medium with additives as diluent, test and control sera are diluted 1:5 and inactivated at 56°C for 30 min. After adding 0.025 ml (1 drop) of medium to each of 12 wells in the row 0.025 ml serum dilution is dropped into the front well. After serial dilutions are made with standardized 0.025 ml microtiter loop diluters 0.05 ml (2 drops) of mycoplasma suspensions containing 10^3 CFU are added to each well. Bring the total volume in each well to 0.20 ml by adding 0.125 ml (5 drops) complete medium to each well. The plates are sealed with tape and incubated at 34°C .

STEP 7. READING AND INTERPRETING THE TEST

The plates are examined daily or at more frequent intervals by placing them over a boxed viewing mirror with a light source above the plates. The accepted endpoints are when color changes are no longer progressive (about 10 days). The growth control well typically changes from red to yellow. The wells of the negative serum control also become yellow while the positive serum control should hold the redness of the pH 7.8 control well out to the known endpoint. A scheme for recording the color changes is as follows: (—)=pH 7.8; (1+)=circa pH 7.3; (2+)=circa pH 6.8; (3+)=circa pH 6.2 or less. That serum dilution which prevents a color change of greater than 50% of that change produced by the organism growth control well is considered the endpoint of the titration. Endpoints may range out to 1:10,240. A four-fold rise in the antibody titer between acute and convalescent phase sera are regarded as significant changes.

Note: An alternate procedure has been studied by Fernald, Clyde and Denny (14). The technic calls for holding serum dilution constant and titrating the antigen. The inhibition titers in their system varied as to sensitivity, but were reproducible within a 4-fold range at the 95% confidence level.

Arginine Utilization Inhibition (AUI) Test

While *M. pneumoniae* and *M. fermentans* antibodies may be titrated by the fermentation inhibition technic, other species of human origin are

excluded from FI testing because they do not ferment carbohydrates. The fact that nonfermentative species utilize arginine with release of ammonia provides a tool for measuring the growth-inhibiting and/or metabolic-inhibiting antibody content of animal and human sera (15). The technique of the test is closely similar to the FI method just covered. The description given below should be helpful in the performance of the AUI test.

STEP 1. PREPARATION OF MEDIUM

The medium used is the complete *M. pneumoniae* fluid medium. Horse serum must be unheated and not free of gamma globulin. Two variations of this medium are prepared as follows: For growing the mycoplasma inoculum, add enough phenol red for a final concentration of 0.002% (0.1 ml of 2% sol./100 ml medium). For use in the antibody titration, add 0.002% phenol red together with 1.0% (w/w) L-arginine HCl and adjust the reaction to pH 7.2. Guinea pig serum is included in the final test system because it has been found to enhance inhibitory activity of homologous antibody while partially suppressing the "breakthrough" of growth that occurs in the higher dilutions during prolonged periods of incubation. The guinea pig serum is introduced via the diluting medium as described in step 6. To each 90 ml complete medium containing arginine and phenol red, add 10 ml unheated guinea pig serum just before use.

STEP 2. PREPARATION OF pH COLOR STANDARDS

The rise in pH in the final test is measured visually by reference to color standards in wells of a microtiter plate incubated along with the specimen under examination. Divide broth medium containing 0.002% phenol red into 16 aliquots of 20 ml each. Using a pH meter, 1 N HCl and 1 N NaOH standards are prepared in 0.2 pH unit increments ranging from pH 5.4 to 9.0.

STEP 3. PREPARATION OF INOCULUM

The mycoplasma species of human origin that liberate ammonia from arginine are: (1) *M. salivarium*; (2) *M. orale* type 1; (3) *M. orale* type 2; and (4) *M. hominis*. The rapidity with which arginine is metabolized apparently varies from species to species and from strain to strain. To select an active metabolizer, it is a good idea to test at least 3 strains of each species. The organisms are grown in 100 ml volumes of complete broth medium containing 0.002% phenol red (no arginine). After 3-4 days of incubation at 34 C, 1.0 ml aliquots

are stored at -70 C to await titration and use in the final test.

STEP 4.

TITRATING MYCOPLASMA INOCULUM

The standard dose of organisms recommended for the test is 10^3 color changing units (ccu). One ccu is defined as the highest dilution of organisms that produces a color change of 0.5 of a pH unit: for example, pH 7.0 changing to pH 7.5. Thaw 1 ampule of the frozen culture from step 3 above. Prepare serial 10-fold dilutions in screw-cap tubes (see Kelton dilution method p. 58) using, for diluent, complete medium containing phenol red and 1% L-arginine. Transfer 0.05 ml of each dilution into wells of a microtiter plate and add 0.15 ml broth medium with phenol red and arginine to each well (total volume=0.20 ml). In another row, add 0.20 ml of each 0.2 increment color standard over the range of 7.0 to 9.0 (11 wells). Into 1 well add 0.20 ml medium diluent and do not inoculate. The plate is then sealed and incubated at 34 C until the maximum pH has been obtained in the titration wells. Read endpoint.

STEP 5. CONTROLLING THE FINAL TEST

As in the FI test, a separate microtiter plate is used for controlling the various reactions of the AUI test. This control plate is incubated along with the antibody titration plates and its preparation is as follows:

- (a) *pH color control*: Add 0.2 ml of each color control from step 2 to wells of the plate. These wells serve as pH indicators for reference with colors obtained in growth control and serum dilution tests. The plastic plate and tape materials may cause a minor decrease in pH as incubation progresses. An alternate procedure is to cover each well with a drop of mineral oil.
- (b) *Mycoplasma growth control*: One well of the microtiter control plate is reserved for observing growth and extent of color change of serum. Add 0.15 ml medium with all supplements, phenol red, and arginine, to 1 of the wells. Use a separate well for each species included in the test. Then add 0.05 ml of mycoplasma suspension. This well should show a rise of pH and darkening of the color. As determined from previous titration (step 4) the pH should rise circa 0.5 of 1 unit, i.e., 7.2 to 7.7 in the ideal test.
- (c) *Medium control*: A single well is reserved for adding 0.2 ml of the same medium dilu-

ent as that being used in the test. This well is not inoculated and should show no pH change except for a possible slightly lowering due to tape and plastic material in the plate. From this well and the growth control well (b above) the extent of pH change produced by the organism is evaluated.

- (d) *Positive serum control*: A serum of known titer is included with each set of tests and it is handled exactly as the test serum. For acceptable results, the endpoint should not vary by more than ± 1 well or dilution.
- (e) *Negative serum control*: A serum known to be free of antibody reactivity is to be included in the control plate. This serum is treated and titrated in the same fashion as test and positive control sera. This serum control serves to detect falsely positive responses in the system.

STEP 6. TITRATING MYCOPLASMA ANTIBODY INHIBITING ARGININE UTILIZATION

Sera from human sources are heat inactivated at 56 C for 30 min without dilution. Rabbit antisera are heat inactivated after being diluted 1:5 using PPLO broth medium without added arginine as the diluent. Drop 0.025 ml broth medium containing all supplements, arginine, and phenol red into wells of a microtiter plate. Then, add 0.025 ml inactivated serum to the first well and serially transfer 0.025 ml across the row of wells using a 0.025 ml microtiter loop. To each well add 0.05 ml mycoplasma suspension containing 10^3 ccu as determined from step 4 above. In order to introduce a final 6.3% concentration of guinea pig serum, add 0.125 ml of diluting medium containing 10% unheated guinea pig serum. The plates are sealed with tape or by dropping mineral oil over each well, and then incubated at 34 C without agitation. Readings are made daily.

STEP 7. READING AND INTERPRETING THE AUI TEST

The plates are examined over a boxed viewing mirror using an overhead light source. Endpoints are recorded when the growth control well shows a rise of 0.5 pH unit over the uninoculated medium control well. For example, comparing the uninoculated well with the color controls shows that the pH is 7.1. In contrast, the inoculated growth control well, by the color standards, shows a pH of 7.6. The serum endpoint is the highest dilution which inhibits 50% of this change, or approximately $\frac{1}{4}$ of a pH unit, when compared with the inoculated well containing organisms growing without antiserum.

Urea Utilization Inhibition Test for Measuring T-Strain Mycoplasma Antibodies

The rising pH which accompanies the hydrolysis of urea provides a means for detecting antibodies to T-strain mycoplasmas. Purcell and colleagues developed a color test for the measurement of these antibodies and demonstrated their presence in human sera derived from various age groups (16). The test is based on the ability of serum antibody to inhibit the changing color of phenol red indicator which occurs during normal growth of T-strains. The method advanced by Purcell, *et al* employs microtiter equipment and is given below.

STEP 1. PREPARATION OF MEDIUM

The broth medium used is a modification of that given on page 17. For T-strain work, thallium acetate is omitted. The broth is supplemented with 1% urea and 0.002% phenol red. The pH is adjusted to 7.2 instead of 7.8. When used as the diluent in the test, a 10% concentration of unheated, pooled guinea pig serum is added to the medium. The guinea pig serum is thought to prevent the "break-through" of ammonia production which sometimes occurs in the presence of antiserum.

STEP 2. PREPARATION OF INOCULUM

The T-960 strain of T-strain mycoplasma isolated from a patient with NGU by Dr. Maurice Shepard has been used successfully in the urea utilization inhibition test. Broth medium containing 1% urea and 0.002% phenol red indicator is seeded with a pure culture using 1-2% inoculum. The optimum pH for T-strain growth is 5.5-6.0. Growth of T-strains is much more rapid than that of classical species and the peak titer is reached in 16 hr. Since broth cultures deteriorate rapidly after the maximum of 10^6 to 10^7 colony-forming units (CFU) has been reached, it is unwise to incubate longer than 16 hr. Aliquots of culture are then stored at -60 C or lower temperatures for further study.

STEP 3. TITRATING THE CCU

Thaw an ampule of stock culture from step 2 above. Prepare serial 10-fold dilutions in supplemented broth medium and transfer 0.2 ml of each dilution 10^2 through 10^8 to wells of a microtiter plate. The plate is sealed and incubated at 37 C until color change becomes no longer progressive. *One ccu is the maximal dilution which produces a color change.* Endpoints to be expected are around 10^5 to 10^6 dilutions of organisms. The number of ccu used in the test is 100/0.05 ml.

STEP 4. CONTROLLING THE TEST

Each time sera are titrated for determining the antibody titer, the following controls must be included:

- (a) 1 row of color standards (see page 49).
- (b) 1 well containing medium only for reference purposes.
- (c) 1 well containing medium plus T-mycoplasmas. This well is observed at frequent intervals for color change. When the color change reaches 0.5 pH unit (as determined from standards), the endpoints are recorded.
- (d) A titration of a positive serum of established titer. The endpoint of this serum should not exceed ± 1 dilution for an acceptable test.
- (e) A titration of a known negative serum is included to detect any falsely positive reactions which might occur in the system.

STEP 5. TITRATING SERUM FOR ANTIBODY CONTENT

Medium containing phenol red and urea is used as the diluent. After the sera have been inactivated at 56 C for 30 min, serial 2-fold dilutions are prepared in microtiter plates. Drop 0.025 ml diluent into the wells. Add 0.025 ml serum to the first well and dilute serially to the end of the row. Then add 0.05 ml seed culture. Add 5 drops (0.125 ml) of the diluent containing 10% unheated guinea pig serum to each well; final volume=0.20 ml. The plates are sealed with tape and incubated at 37 C.

STEP 6. READING AND INTERPRETATION OF TEST

The endpoints are recorded when the control well containing mycoplasma culture plus medium shows a color change of 0.5 of a pH unit (usually 2 days). This determination is made by comparing colors of medium control with mycoplasma control while referring to the row of color standards which progress in increments of 0.2 pH units. The titer of the serum is the greatest dilution which inhibits 50% of the color change or approximately 0.25 pH unit. A second reading may be made when the control well progresses further to a change of 0.8 pH unit. With both readings recorded, the acceptable endpoint is the higher of the 2 readings. Titer of patients with NGU range to 1:128 (16).

Hemagglutination Inhibition (HI) Test

In 1965, Feldman and Suhs described *M. pneumoniae* hemagglutination (HA) and its inhibition by antibody (17). Later, they applied their test to seroepidemiologic studies in various populations (18). They detected no HI antibody in normal family members under the age of 15. In the elderly, 53% were positive by their criteria. Point Barrow, Alaska residents were 68% positive and above age 15 they were 90% positive. It was noted with interest that HI antibody to *M. pneumoniae* could persist for 10 years in undiminished titers. It was concluded by these workers that the HI test is at least as sensitive as the CF test for measuring antibodies to *M. pneumoniae*.

The Feldman-Suhs HI test is easily performed by macro or micro methods. The HI tests have been made in RB-96 disposable trays, clear plastic trays (Linbro); glass tubes 12×75 mm may be used also. Microtiter plates and loop diluters are adaptable; however, instead of 0.1% erythrocytes it has been found necessary to use 0.2% concentration of erythrocytes in phosphate buffered saline (PBS) with 0.4% gelatin if microtiter plates are used. Two requirements that differ from other HI tests are unusual: (1) The erythrocytes to be agglutinated must first adsorb antibodies directed toward themselves from a normal serum. The serum of choice that provides this antibody is that pooled from the horse; (2) The HA antigen must be a viable broth culture of *M. pneumoniae*. Other features of the test indicate that either vervet monkey, rabbit, or human erythrocytes can be used. The addition of 0.2% gelatin to the saline diluent accelerates the settling of erythrocytes. While room temperature incubation is as good as 37 C incubation, a temperature of 4 C is to be avoided.

STEP 1. PREPARING *M. pneumoniae* HEMAGGLUTININ

The hemagglutinin is a broth culture of *M. pneumoniae* grown in the medium described on page 17. Screw-cap tubes of the fluid medium are inoculated with a 4-day broth culture of the organism using 1 part inoculum in 320 parts medium. The cultures are incubated at 37 C until the hemagglutinin reaches its maximum titer, i.e., circa 7 days. The hemagglutinin appears later than multiplication of the mycoplasmas and falls off sharply once the peak of growth is reached. Daily titrations of the broth cultures can be made as outlined in step 2. In the hands of Feldman and Suhs, a maximal titer of 1:320 was reached on the 7th day of incubation. The broth tubes can be

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stored frozen at -65°C for long intervals of time without deleterious effects.

STEP 2. TITRATING THE HA UNIT

Hemagglutination inhibition determinations are made by titrating antibody against 2 HA units of antigen per tube, well or serum dilution. The HA unit is first determined as follows: Thaw a frozen antigen culture rapidly by immersing the tube in a 37°C water bath. Prepare a 1:5 dilution of antigen by adding 0.2 ml to 0.8 ml of 30% horse serum-phosphate buffered saline with gelatin (HS PSGB). This 1:5 dilution is then allowed to incubate at room temperature for 60 to 90 min. Prepare serial 2-fold dilutions in 30% HS PBSG. To each tube or well add an equal volume of 0.1% red cells (in HS PBSG). Shake to mix and allow to incubate at room temperature. The endpoints are recorded after approximately 3 hr when the cell control shows a well-formed button. *The cell control consists of equal volumes of 30% HS PBSG and 0.1% red cell suspension incubated in the absence of antigen.* One HA unit is defined as the greatest dilution of antigen which shows 4+ agglutination of cells.

VOLUMES OF REAGENTS FOR HA TITRATION

Reagents	Equipment		
	Glass tubes	Automatic	Microtiter
Antigen dilution	0.5 ml	0.25 ml	0.05 ml
Erythrocytes	0.5 of .1%	0.25 of .1%	0.05 of .2%*
TOTAL	1.0	0.50	0.10

STEP 3. TITRATING HI ANTIBODY

All sera, whether of human or animal origin, are inactivated at 56°C for 30 min. Only 0.2 ml of undiluted specimen are usually heated. Serial 2-fold dilutions of serum are then made in 30% HS PBSG diluent. Volumes to use are summarized in the table below. The initial dilution in the first tube or well is 1:2. When dilutions are completed, they are allowed to stand for 30 min before adding an equal volume of erythrocyte suspension. The test is then incubated for 3 hr at room temperature or until the cell controls are completely down. The titer of the serum is the highest initial dilution in which less than 2+ hemagglutination is present.

VOLUMES OF REAGENTS FOR HI TITRATION

Reagent	Equipment		
	Glass tubes	Automatic	Microtiter
Serum dilution	0.25 ml	0.125 ml	0.025 ml
8 units antigen	0.25	0.125	0.025
Erythrocytes	0.50 of .1%	0.25 of .1%	0.050 of .2%*
TOTAL	1.00	0.500	0.100

* The .2% concentration is prepared in PBS containing .4% gelatin.

STEP 4. CONTROLLING THE HI TEST

A total of 4 controls should be included with each set of determinations. A cell control consisting of erythrocyte suspension and diluent in equal volumes provides a guide on when to read the test, and rules out false positive hemagglutination. A titration of antigen should be included to insure that 2 units are present in the test. A known positive serum endpoint should vary no more than ± 1 dilution from the established titer while the negative serum control should show only buttons and no false agglutination.

STEP 5. INTERPRETATION AND SIGNIFICANCE OF HI TITERS

Any serum showing a titer of 1:2 or greater is considered positive. Titers range up to 1:1024 in naval recruits (18). Four-fold or greater increases in titer are regarded as diagnostic of *M. pneumoniae* infection.

Indirect Hemagglutination Test

It has been demonstrated by Dowdle and Robinson that the sera of patients with *M. pneumoniae* infection agglutinate sheep erythrocytes which have been treated with tannic acid and then sensitized with *M. pneumoniae* antigen (19). The test is specific and at least as sensitive as the complement fixation (CF) test. The method outlined below is that of Dowdle and Robinson with minor modifications.

STEP 1. PREPARING THE ANTIGEN

Using a 10% inoculum, seed the standard broth medium with a 4-5 day old starter culture of *M. pneumoniae* and allow to incubate 15 days. To reduce the volume of fluid and facilitate high speed centrifugation, dialyze the culture against polyethylene glycol until 1/5 of the original volume remains. Collect the organisms in a Spinco ultracentrifuge spinning at 30,000 rpm for 1 hr. The pellet is resuspended in pH 7.2 phosphate buffered saline to 1/2 the original volume and again subjected to centrifugation. The once-washed organisms are then resuspended in phosphate buffered saline to 1/20 the volume of the original broth culture, i.e., 50 ml/L. The final suspension is then treated in a sonic oscillator for 10 min before sensitizing the erythrocytes.

STEP 2. TREATING SHEEP ERYTHROCYTES WITH TANNIC ACID

Sheep erythrocytes in Alsever's solution are collected by centrifugation and washed 3 times in pH

7.2 phosphate buffered saline. They are resuspended in the buffer to a final concentration of 2.5% and then mixed with an equal volume of 1:20,000 tannic acid solution*. The flask is then placed in a 37 C incubator or water bath for 10 min. The cells are then collected in a centrifuge and washed once in phosphate buffered saline. Finally, the erythrocytes are resuspended in pH 6.4 phosphate buffered saline to a concentration of 2.5%.

STEP 3. PREPARE RABBIT SERUM DILUENT

A rabbit serum diluent is used throughout the final test. To each 149 ml of pH 7.2 phosphate buffered saline add 1 ml of inactivated, adsorbed rabbit serum (see page 13). Store at 4 C. Normal horse serum may be substituted for rabbit serum.

STEP 4. SENSITIZING ERYTHROCYTES WITH ANTIGEN

Using pH 6.4 phosphate buffered saline, dilute the 2.5% suspension of erythrocytes 1:5. To 1 volume of this 0.5% suspension of cells, add 1 volume of antigen, and incubate 30 min at room temperature. The sensitized erythrocytes are collected in a centrifuge and washed twice with rabbit serum diluent. In the test, sensitized cells are used in 0.5% concentration with rabbit serum diluent.

STEP 5. CONTROLLING THE TEST

Each set of antibody titrations requires the following controls:

- (a) The first 3 dilutions of test serum, plus tannic acid-treated cells to show that patient sera do not react falsely positive, are included. These 3 dilutions are incorporated in the last 3 wells in each row.
- (b) A serum of previously established titer is included in one row. This serum is treated exactly as the unknown sera.
- (c) A control well of sensitized erythrocytes plus diluent is included to show that the rabbit serum diluent causes no agglutination.

STEP 6. TITRATING *M. pneumoniae* HI ANTIBODY

Microtiter plates with "U" shaped wells may be utilized for the final test.

- (a) Add 0.05 ml rabbit serum diluent to all but the 1st and 10th well.
- (b) Add 0.1 ml 1:10 serum dilution to 1st and 10th well in row (last 3 wells are controls).

* Tannic acid concentration may have to be adjusted, using higher dilutions.

- (c) Serially transfer 0.05 ml from 1st to 10th well.
- (d) Serially transfer 0.05 ml from 10th through 12th well (controls).
- (e) Add 0.05 ml of 0.5% suspension of sensitized erythrocytes to all test wells.
- (f) Cover test plate with empty plate and incubate at 35 C.

STEP 7. READING AND INTERPRETING THE TEST

The endpoints are recorded when the diluent control and the negative serum control show well-formed buttons. Usually, buttons appear in 2-3 hrs. The endpoint of the titration is the greatest dilution of serum which shows a clearly positive agglutination of the erythrocytes. The diluent control well should show a button. The positive control serum should show button formation within 1 dilution of the established titer. The first 3 serum dilutions plus tannic acid-treated cells should show no agglutination. A 4-fold (2 well) rise in titer is regarded as being a significant change.

Immunodiffusion (ID) Test

A rapid and easy immunodiffusion or double diffusion gel precipitation test for detecting antibody to *M. pneumoniae* in human sera has been recommended by Conant, Somerson, and Senterfit (7). In their hands, the ID test and the tetrazolium reduction inhibition (TRI) test gave excellent agreement with patient sera and sera from *M. pneumoniae* vaccinees. This result strongly suggests that the ID test measures the same antibody as the TRI test and that the simpler ID reaction could be advantageously used as a qualitative test for detecting persons with *M. pneumoniae* antibody. The antigen for this test is prepared from organisms grown on the surface of glass (see page 42). In order to achieve a higher yield of antigen a special medium containing HEPES buffer is used (see page 16).

STEP 1. PREPARING THE ANTIGEN

Inoculate 0.1 ml suspension of *M. pneumoniae* into 25 ml of medium in a 6 fl. oz. prescription bottle. Incubate bottle at 37 C in horizontal position. After 4 days decant the medium and wash 4 times with 10 ml phosphate buffered saline, pH 7.4. Then add 0.4 ml distilled water and remove the organisms from the glass with a rubber policeman. To obtain 4.0 ml antigen (approximately 1100 tests) process 10 bottles of medium as described. When all have been harvested, pool the suspensions. The pooled material is then frozen and thawed 10 times. Such antigen

preparations have been reported to be active in ID tests for as long as 6 months if stored at 4 C.

STEP 2. DOUBLE DIFFUSION GEL PRECIPITATION (ID)

A highly purified agar is used. Agarose 0.6% in 0.15 M NaCl, or Ionagar No. 2, 0.8% in 0.15 M NaCl is poured onto a 1"×3" glass slide and allowed to harden. Wells for antigen and sera are cut with a gel punch having a die that cuts 4 mm holes 2–3 mm apart in a circular pattern. The center antigen well is usually over 5 mm in diameter. For testing animal sera 3 mm wells are recommended.

The center, antigen well, and the antiserum wells are filled using antigen and sera undiluted. The slides are incubated at 33 C in a very humid atmosphere to prevent evaporation. One method is to place the ID slide in a Petri dish containing a moistened piece of filter paper. An alternate method is to place many slides in a humidified chamber. At 33 C, the usual period of incubation needed before precipitin lines develop is 5 to 16 hr with agarose medium. A known positive control serum and a serum of known negativity is included with each set of tests to insure that the antigen is reactive and that falsely positive precipitin bands are not produced.

STEP 3. READING THE ID TEST

A positive reaction is indicated by the appearance of a single band between antigen and antisera, and a negative response is indicated by the absence of a band. While certain sera of high titer will show 2 bands, the predominant band gives the reaction of identity with its neighbor band if that too is positive, i.e., the bands coalesce instead of crossing.

Cold Hemagglutinin Test

The development of cold hemagglutinins in the sera of many patients with primary atypical pneumonia was noted by Peterson, Ham and Finland in 1942 (20). It was subsequently shown that *M. pneumoniae* infection and cold agglutinins were associated phenomena in primary atypical pneumonia (21, 22, 23). Since the cold agglutinin titer is of some use in the diagnosis of primary atypical pneumonia and has value as a research tool, a procedure for titrating these antibodies is given.

I. QUANTITATIVE TEST

STEP 1. COLLECTION OF SPECIMEN

The serum is obtained by allowing the blood specimen to clot at 37 C preferably in a water bath.

Since this hemagglutinin reacts only in the cold it does not adsorb to patients red blood cells if clotting proceeds at 37 C. The serum is stored frozen at –20 C until tested. It is customary to titrate acute phase and convalescent phase sera side by side in the same test. The second serial blood is usually drawn from the patient 14 days after the first specimen.

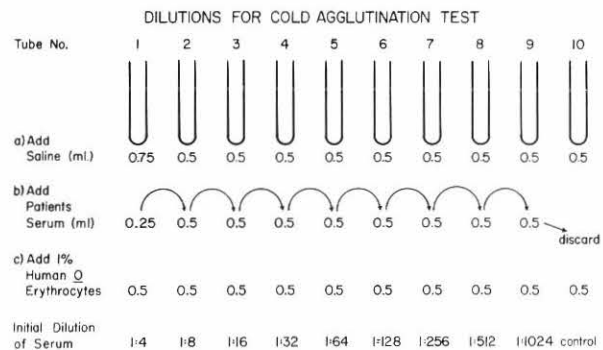


Fig. 32. Scheme of titrating cold hemagglutinating antibodies in patient sera.

STEP 2. TITRATING SERUM

Place 10 tubes in a rack for each serum to be tested. To the first tube add 0.75 ml normal saline solution and to all others add 0.50 ml. Add 0.25 ml unheated serum to the 0.75 ml saline in the first tube making a 1:4 dilution. Transfer 0.5 ml serially and discard 0.5 ml from the 10th tube. Add 0.5 ml 1% suspension human type O erythrocytes to all tubes. Shake rack and keep at 4 C overnight. Controls: A positive serum of known titer and a negative serum are included with each test. They are treated exactly as the unknown serum above. (Fig. 32)

STEP 3. ENDPOINT DETERMINATION

The tubes are examined for definite floccular agglutination under artificial light using the naked eye. The examination is made after the tubes have been inverted 3 times. The last tube in the dilution series showing visible clumping is considered the endpoint.

STEP 4. CONFIRMING COLD AGGLUTINATION

Place all positive tubes in a 37 C water bath for 1 hr. Examine again for presence of agglutination. True cold agglutinins disappear after being warmed for this length of time.

II. RAPID SCREENING TEST FOR COLD AGGLUTININS IN PNEUMONIA

In 1958 Garrow devised a rapid screening test for the presence of increased cold agglutinins (24).

Cordero, *et al*, utilizing this test, reported that it was successful in detecting approximately 75% of pneumonia due to *M. pneumoniae* (25). Griffin demonstrated that the rapid screening test was sensitive at a titer of 1:64 or greater, and estimated from the rarity of false-negative reactions that the sensitivity was 98% and the specificity approached 100% (26).

PROCEDURE OF GRIFFIN

Whole blood is obtained by lancet puncture of the finger. Four to five drops of blood are collected in a 6×70 mm Wasserman tube containing 0.20 ml of 3.8% sodium citrate solution. The tube is corked and placed in the corner of a refrigerator ice cube tray containing water (0–4°C). After 1½ minute the tube is tilted on its side allowing the blood to run down the length of the chilled tube. A positive test is evidenced by definite flocculation and all other appearances are called negative.

Streptococcus MG Agglutinins

In 1943 Thomas and co-workers demonstrated a non-hemolytic streptococcus in the lungs of patients who had expired from primary atypical pneumonia (27). This organism, designated *Streptococcus* MG, was shown to be of etiological significance in a proportion of patients with pneumonia and milder respiratory infections. The development of agglutinins against this streptococcus was also shown in a higher proportion of patients with pneumonia than with other conditions or from healthy individuals. These findings of the early 1940's were not confirmed by others, and interest languished on the possible role of *Streptococcus* MG in pneumonia. However, interest was aroused once more by the knowledge that *M. pneumoniae* is associated with this condition. Pease, in England, asserted that *M. pneumoniae* is the L-form of *Streptococcus* MG (28). McGee and associates demonstrated that *M. pneumoniae* and *Streptococcus* MG are not genetically related (29). Lind could not demonstrate that *Streptococcus* MG organisms were associated with pneumonia (30). The development of *Streptococcus* MG agglutinins in patient sera are now thought to be due to the cross reacting antigens of *M. pneumoniae*. This concept is substantiated by the work of Marmion, *et al*, and by Lind, who have shown that *Streptococcus* MG and *M. pneumoniae* have antigens in common (30, 31). The *Streptococcus* MG agglutinin titer of serum is still of interest and can be determined as described below.

STEP 1. SERUM AND ANTIGEN

Patient sera under examination for *Streptococcus* MG agglutinins are not heated. The *Streptococcus*

MG used for antigen is prepared by growing the organism in Todd-Hewitt broth for 18 hr. The cells are washed 3 times and suspended in 0.9% NaCl solution. The amount of saline to use is the quantity which gives a turbidity equal to tube No. 5 on the McFarland scale. Usually this volume is equal to one-third to one-half of the original culture. The bacteria are then killed by heating at 65°C for 1 hr. Merthiolate is then added in a final concentration of 1:10,000 as a preservative. Suspensions prepared in this manner may be kept for 3 to 4 months in the refrigerator. *Streptococcus* MG agglutinating antigen is also available on the market. When this material is used it must be diluted according to the instructions on the label before adding to the test.

STEP 2. TITRATING THE SERUM

Place 8 tubes in a rack for each serum to be tested. To the first tube add 0.8 ml physiological saline. Add 0.5 ml to each of the remaining tubes. Transfer 0.2 ml undiluted serum to first tube making one ml of 1:5. Mix and transfer 0.5 ml through the 7th tube discarding 0.5 ml. The eighth tube serves as a control on autoagglutination in saline. Mix and dilute antigen. Add 0.5 ml to each tube in the test. The final dilutions are now 1:10 to 1:640. A control positive serum of known titer is included with the test, and is treated exactly as the test serum. See Fig. 33 for scheme.

STEP 3. INCUBATION

Two methods of incubation have been prescribed. After shaking the rack, place in water bath at 37°C for 2 hr, then overnight in the refrigerator, and finally another 2 hr at 37°C. An alternate method which is simpler and acceptable is to incubate at room temperature overnight.

DILUTIONS FOR STREPTOCOCCUS MG AGGLUTINATION TEST

TUBE No.	1	2	3	4	5	6	7	8
a) Add Saline (ml)	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5
b) Add Patients Serum (ml)	0.2	0.5	0.5	0.5	0.5	0.5	0.5	discard
c) Add Strep MG Antigen (diluted ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Final Volume (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final dilution of Serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Antigen Control

Fig. 33. Scheme of titrating *Streptococcus* MG agglutinins in patient sera.

STEP 4. ENDPOINT DETERMINATION

The tubes are read as follows:

- 4+ Complete agglutination with a solid disc of streptococci. Clear supernatant diluent.
- 3+ Agglutination shows large clumps. Clear supernatant, but incomplete settling of bacteria at bottom of tube.
- 2+ Incomplete agglutination. Clearing of supernatant fluid.
- 1+ Agglutination shows turbid fluid, but with aggregates visible to the naked eye.

A positive reaction is the highest dilution in which reactions of 1+ or more are observed. Titers above 1:20 are rarely found in normal persons.

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VIABLE PLATE COUNTS

The quantitative determination of mycoplasma organisms in fluid cultures is essential for the construction of growth curves and for evaluating factors necessary for improving media. Knowledge of population densities is furthermore essential to immunologists who seek maximum growth when preparing antigens for serological or immunizing purposes. While turbidometric methods have been described for enumerating mycoplasma organisms, it is generally conceded that plate counts are more accurate and dependable (1). Kelton described a counting technique which is accurate and easily performed (2). It requires no special equipment or complex manipulations. Kelton's technique, with minor modifications, is as follows:

Using screw-cap tubes, 10-fold dilutions of fluid culture are prepared by serially transferring 0.50 ml to a diluent of 4.5 ml sterile 0.5% solution of Difco Proteose Peptone No. 3 in distilled water (Fig. 34).

One-tenth ml of each dilution tube is then spread over the surface of duplicate agar plates. Laboratory adapted strains that grow rapidly may be allowed to incubate 72 hr. Freshly isolated strains that grow slowly, or grow only under anaerobic conditions,

may require 7 to 14 days of incubation before the colonies in the agar attain maximal growth.

In order to facilitate the enumeration of all colonies with the aid of the microscope and to avoid errors of duplication, a series of closely spaced parallel lines is drawn with a felt pen and transparent ink (Fig. 35).

With the plate inverted under a dissecting microscope, the colonies under the lines are plainly visible at 30X magnification and up to 1,000 colonies can be counted without difficulty. With allowance for strain variation the growth curves of mycoplasma are similar to those obtained from bacteria. Although the log phase has been found to vary with the culture studied, the logarithmic phase of growth typically follows a straight line. The maximal number of colony-forming units to expect of strains that grow well is usually 10^9 /ml.

It is known that *M. pneumoniae* produces microcolonies or clumps of organisms in fluid medium. Hence, plate counts may show considerable variation depending upon age of the culture, agitation of the flasks, and other poorly understood factors. Low and Eaton have reported increased numbers of *M. pneumoniae* in shaker cultures but attributed their results to aeration (3).

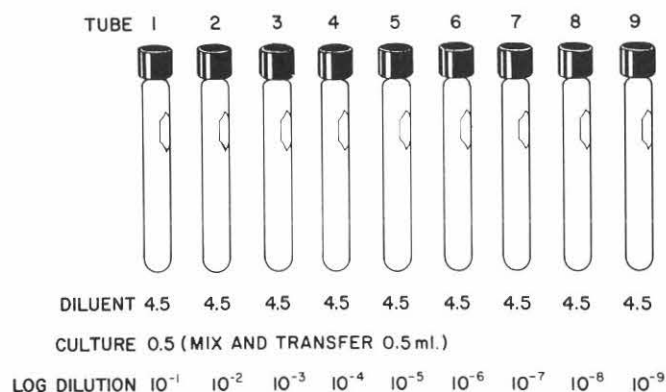


Fig. 34. Dilution scheme for performing plate counts on mycoplasma species.

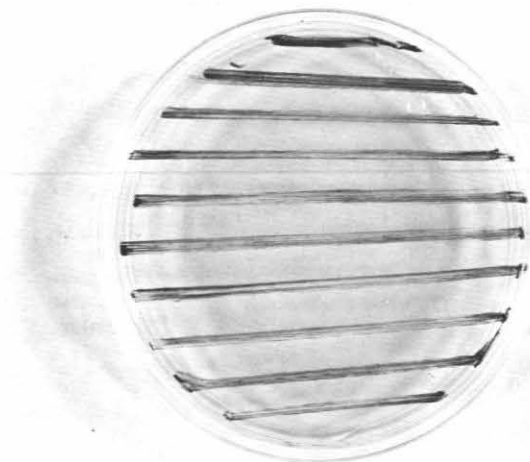


Fig. 35. A plate marked with transparent ink to aid in enumeration of mycoplasma colonies under the microscope.

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STORAGE AND MAILING OF MYCOPLASMA CULTURES

Kelton reported on the viability of mycoplasma strains after lyophilization and storage at various temperatures (1). He lyophilized broth cultures that were mixed with equal amounts of sterile skim milk. He also stored broth cultures at -5°C , at -26°C and at -65°C over prolonged periods to assess the survival of the organisms. Kelton's observations are summarized below:

Storage of 26 Mycoplasma Strains of Avian, Animal, Human and Saprophytic Origin

Stored after Lyophilization	Frozen Broth
All 26 strains remained viable when stored at -26°C for 2 years and then at -65°C for the balance of a 3- to 4- year period	After 10 to 18 months, all but 1 strain were viable at -26°C . After 12 months, all were viable at -65°C . At -5°C viability of most strains declined rapidly. Greatest losses in numbers occurred after the first freezing.

In addition to the experience of Kelton, above, Tully and Ruchman reported recovering viable mycoplasmas from 20-year old lyophilized cultures received from Sabin (2). Morton described freeze-

drying of mycoplasmas in 1963 (3). Andrews lyophilized mycoplasmas with glucose and sucrose as additives (4). More recently Norman, Franck and Choate reported on 9 years of preservation of mycoplasmas by freezing in liquid nitrogen and lyophilization with sucrose at the American Type Culture Collection (5).

In the writer's laboratory 3 ml of broth in a screw-cap vial are inoculated by agar block. After 4 days of incubation at 37°C the vial is placed at -60 to -70°F . Such material has remained viable for 6 years.

When a mycoplasma culture is to be mailed packaging with dry ice is unnecessary. Instead, send an appropriate screw-cap vial containing frozen broth, or emulsified agar cultures packaged in a mailing container and post "AIRMAIL SPECIAL DELIVERY." The culture soon thaws but the organism remains viable and will be subcultured with ease. For example, cultures mailed from Great Lakes, Illinois to Egypt, Alaska, Hawaii, and Japan have been subcultivated successfully. The soft agar used in mycoplasma plates cannot be expected to withstand the stresses of mail handling.

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SOURCE OF SUPPLIES

ITEM	MANUFACTURER	ITEM	MANUFACTURER
Agamma Horse Serum	North American Biologicals Inc. North Miami, Fla. 33161	Mycoplasma Antigens (For all human species)	BBL, Div. of Bioquest Cockeysville, Md. 21030
Agarose, special grade	Mann Research Labs Div. of Becton Dickinson New, York, N.Y. 10006	Mycoplasma Antisera (For all human species)	BBL, Div. of Bioquest Cockeysville, Md. 21030
Amphotericin B (Fungizone), 50 mg	E. R. Squibb & Sons New York, N.Y. 10022	Mycoplasma Enrichment, lyophilized (Eaton Agent Enrichment)	BBL, Div. of Bioquest Cockeysville, Md. 21030
Ampul, 1 ml size, "Neutraglass"	Standard Scientific Chicago, Ill. 60626	Mycoplasma pneumoniae CF Antigen	BBL, Div. of Bioquest Cockeysville, Md. 21030
L-Arginine Hydrochloride	Calbiochem Los Angeles, Calif. 90054	Multidisk, 8-tipped	Colab Laboratories Inc. Glenwood, Ill. 60425
Azure II, N.F.	National Analine Div. Allied Chemical & Dye Corp. New York, N.Y. 10006	Nitrogen Cylinder with 95% Nitrogen and 5% Carbon Dioxide	National Cylinder Gas Co. Milwaukee, Wis. 53203
Bovine Serum Albumin (Fraction V)	Armour Pharmaceutical Kankakee, Ill. 60901	Penicillin G, Potassium, for Injection, 1,000,000 units	Chas. Pfizer & Co. New York, N.Y. 10017
Complement, Bacto	Difco Laboratories Detroit, Mich. 48201	Petri Dish, Plastic, 60 x 20 mm	Lab-Tech Products Westmont, Ill. 60550
Cresol Red, Indicator	Matheson Coleman & Bell Los Angeles, Calif. 90053	Petri Dish, Plastic, 100 x 15 mm	Lab-Tech Products Westmont, Ill. 60559
Dextrose Solution, 10%	Difco Laboratories Detroit, Mich. 48201	Phenol Red, USP	National Analine Div. Allied Chemical & Dye Corp. New York, N.Y. 10006
Dienes Special Stain	North American Biologicals Inc. North Miami, Fla. 33161	Povitsky Bottle	Standard Scientific Chicago, Ill. 60626
Dimethyl Barbituric Acid	Mallinckrodt Chemical Works St. Louis, Mo. 63160	PPLO Agar	Difco Laboratories Detroit, Mich. 48201
Disc, Sensitivity Test (SENSI-DISC)	BBL, Div. of Bioquest Cockeysville, Md. 21030	PPLO Broth	Difco Laboratories Detroit, Mich. 48201
Eagle's Minimum Essential Medium (10X)	Grand Island Biological Co. Grand Island, N.Y. 14702	Pump, high-vacuum, Welch, Duoseal	Scientific Products Waukegan, Ill. 60086
Gel Punch, Type 6808A	LKB Instruments Inc. Rockville, Md. 20852	Sodium diethyl barbiturate	Fisher Scientific Co. Fair Lawn, N.J. 07410
Heater, Paraffin, Embedding	Standard Scientific Chicago, Ill. 60626	Soy peptone powder	Sheffield Chemical Co. Norwich, N.Y. 13815
HEPES (N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid)	Calbiochem Los Angeles, Calif. 90054	Streptococcus MG Antigen	Colab Laboratories Inc. Glenwood, Ill. 60425
Hemlysin, Bacto, anti-sheep, glycerinated	Difco Laboratories Detroit, Mich. 48201	Streptococcus MG Control Antiserum	Colab Laboratories Inc. Glenwood, Illinois 60425
Horse Serum, Normal 100 ml bottle	North American Biologicals Inc. North Miami, Fla. 33161	2,3,5-triphenyl-2H- tetrazolium chloride	Eastman Organic Chemicals Rochester, N.Y. 14601
Ionagar No. 2	Colab Laboratories Inc. Glenwood, Ill. 60425	Thallium acetate	Fisher Scientific Co. Fair Lawn, N.Y. 07410
Jar, Anaerobic, and covers	Pfizer Diagnostics Inc. Chicago, Ill. 60622	Tryptic digest broth powder (Fields)	BBL, Div. of Bioquest Cockeysville, Md. 21030
Methylene Blue, Bacto	Difco Laboratories Detroit, Mich. 48201	Trypticase soy broth	BBL, Div. of Bioquest Cockeysville, Md. 21030
Microtiter Dropper, 0.025 ml	Cook Engineering Co. Alexandria, Va. 22314	Vacuum pump, "Little Giant," 1/2 H.P.	Gelman Instrument Co. Ann Arbor, Mich. 48106
Microtiter Loop Diluter, 0.025 ml	Cook Engineering Co. Alexandria, Va. 22314	Wax, paraffin, tech	Fisher Scientific Co. Chicago, Ill. 60651
Microtiter Plates	Cook Engineering Co. Alexandria, Va. 22314	Yeast autolysate	Albimi Laboratories Flushing, N.Y. 11354
		Yeast extract, fresh	Microbiological Associates Bethesda, Md. 20014
		Yeast, pure dry, Fleishman's type 20-40	Standard Brands Inc. New York, N.Y. 10022

